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ON THE PRENATAL GROWTH OF THE MAMMARY GLAND RUDIMENT IN THE MOUSE

By B. I. BALINSKY*

Department of Zoology, University of the Witwatersrand, Johannesburg

The development of the mammary glands has attracted much attention from morphologists, embryologists and physiologists. For a review of the literature on the subject the reader is referred to Turner (1939) and Speert (1948). The postnatal development, especially, has been studied with great success and the factors controlling the growth and differentiation of the glands have been investigated in great detail. The prenatal development, on the other hand, has not been subjected to investigation by quantitative methods. In particular, the processes of cell proliferation, and the growth of the early rudiments of the mammary glands have not been studied, so far as I have been able to discover. The present work is an attempt to fill this gap.

MATERIAL AND METHODS

Mice of a heterogeneous population were used for this investigation, and no attempt was made to select embryos of a special genotype. Embryos of known age, from the 8th day to birth, were studied. The specimens were fixed in Bouin's fluid, embedded in paraffin, cut transversely and stained with haematoxylin alone or with haematoxylin and eosin.

The growth of the mammary glands was estimated from the volume, obtained by drawing every section of a number of glands with a camera lucida and then measuring the surface of the section with a planimeter. Adding up the surface area of all the sections gave the volume in arbitrary units. The rate of increase of the gland volume was then compared with the growth of the whole embryo. To determine the latter several embryos for every day of development were weighed, starting with 9-day-old embryos. The figures obtained give only a very crude approximation and no attempt has been made either to exclude the variability of the embryos, which seems to be very considerable, or to treat the results statistically.

For the investigation of the cellular proliferation in the mammary gland rudiments extensive use was made of the mitotic index method. The cellular proliferation is, of course, proportional to the growth rate only under certain conditions, especially if the size of the cells does not change to a great degree and if there is no loss of cells. Both these conditions apply to the mammary glands at least approximately. The mitotic index in early mammary gland rudiments was compared with the mitotic index in the epidermis, as a tissue from which the mammary glands are derived and which therefore is a convenient standard against which the mitotic activity of the glands can be tested. Accordingly, an approximately equal number of epidermis cells was counted in the immediate vicinity of each mammary gland used for mitosis counts. In the case of late mammary gland rudiments (age over 15 days) there was no point in comparing the mitotic activity of mammary gland cells and

* This work was done while the author was at the Animal Breeding and Genetics Research Organization at the Institute of Animal Genetics, Edinburgh University.

epidermis cells because of the obvious divergence in their differentiation. The mitotic index of these glands was therefore compared with the index in earlier mammary gland rudiments. Besides this, counts were made in distal and in proximal parts of the late mammary glands and the mitotic indices of these parts compared. All the cells of early mammary gland rudiments were counted, but in the very much larger glands of later stages this could not be done, and only a part of the sections of each gland was used for the counts. Only those cells were counted as mitoses in which there was no nuclear membrane, that is, metaphases, anaphases, and the earliest telophases with an irregular contour of the daughter nuclei. The subjective factor in the evaluation of the early prophases is thus excluded.

The method of determining the mitotic index has its defects and dangers which have not been fully realized by investigators using this method. One danger lies in the evaluation of the statistical significance which can be attached to the figures obtained. It has been pointed out by Pasteels (1940) that the method has been applied uncritically and that, in fact, conclusions have been drawn from material that is statistically inadequate. I have checked Pasteels' conclusions and found his criticism quite correct. The formula to be applied for the calculation of the standard error of the mean of the mitotic index of a single sample is the following:

$$s_m = \sqrt{\frac{pq}{N}},$$

where $p =$ percentage of dividing cells ('mitotic index'), $q = 1 - p$ and N is the total number of cells counted. Applying this formula, and taking into consideration the actual values of the mitotic indices observed, it was found that it is necessary to count several thousands of cells in order to discover a statistically significant difference in the mitotic indices of different tissues. This has a direct bearing on the determination of the mitotic index in mammary glands as the number of cells in the early mammary gland rudiments is less than a thousand. Even a complete count of all the cells in one mammary gland rudiment is therefore insufficient for the results to be statistically significant.

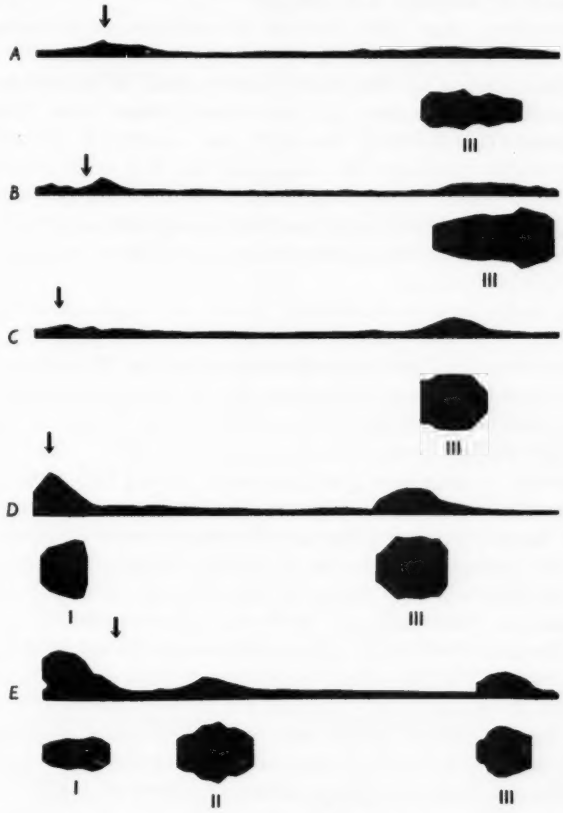
If the mitosis counts are made in several different embryos or in several different glands of the same embryo the question arises whether it is legitimate to treat such data as samples of one and the same binomial distribution. The significance test to be applied to such data depends on whether the difference between the tissues which are to be compared is the same for all samples (in statistical terms, whether there is interaction as regards the mitotic index between sample and tissue). Such data may be analysed by a treatment of the differences within samples, with appropriate weighting. This method as applied here is the same as that given by Snedecor (1946, p. 291), but for enumeration rather than measurement data.

DATA ON THE MORPHOLOGY OF THE DEVELOPING GLANDS

It has been necessary to re-investigate the morphological changes in the mammary gland rudiments as a background for the investigation of growth and proliferation. The claim by Turner & Gomez (1933) to have found a mammary line in 13-day-old mouse embryos could not be confirmed. No such line could be discovered in the embryos studied. The mammary gland rudiments seemed to make their appearance

independently of one another. There is some variation in the time when the first mammary gland rudiments can be discerned, but usually they could be found in 11-day-old embryos.

As it is sometimes difficult to visualize the form and exact extent of structures from serial sections, graphic reconstructions were prepared with the aid of a camera lucida of the epidermal thickenings of several embryos at the time when the mammary



Text-fig. 1. Graphic reconstruction of epidermal thickenings on the body side during the formation of the mammary gland rudiments. Continuous black line—greatest thickness of skin epidermis. Black spots—multilayered thickenings (mammary gland rudiments) in surface view. I, II, III—numbers of the glands. Arrowheads show the posterior margin of the forelimb. The five cases (*A–E*) represented are arranged in order of increasing differentiation. The irregular broadening of the line representing the thickness of the epidermis in the region of the forelimb (on left) is partly due to the sections going oblique to the surface of the epidermis.

gland rudiments first become visible. These reconstructions are presented in Text-fig. 1. The maximal thickness of the epidermis along the side of the body in each section is represented in the drawing by a continuous line. The thickened parts of the epidermis where it has become multilayered are represented as black areas. The

latter are shown in surface view. Both have been drawn to the same scale. Only the region of the three pectoral glands has been shown, and the arrows indicate the position of the posterior border of the forelimb in each case. The uppermost reconstruction (*A*) represents an advanced 10-day-old embryo, the earliest in which a distinct epidermal thickening has been found. The rest represent 11-day-old embryos arranged in order of increasing differentiation. The individual glands here and later will be referred to by numbers, I, II and III.

The reconstructions show that there is no continuous longitudinal thickening from which the individual glands are derived. It is remarkable that the glands do not appear simultaneously; in the first four embryos gland III is already distinct, but gland II appears only in embryo *E*. The reconstructions show further that the rudiment of gland III, when first discernible, has a distinctly elongated form, but this is the only feature suggestive of a mammary line that could be found. In a few hours the rudiment ceases to be elongated and becomes oval or round. In the development of gland II, as clearly seen from the reconstruction, there is no elongated thickening preceding the formation of the rudiment or linking it up with the rudiments I and III.

The earliest rudiments, as mentioned above, are represented by very slight thickenings. Pl. 1, fig. 1, shows a section of the rudiment of gland II in the youngest embryo (*A*) which was used for the graphic reconstruction. The cells of the epidermis have scarcely begun to arrange themselves into a multilayered thickening. Pl. 1, fig. 2, shows a section of the next youngest embryo (*B*). In this case the thickening is still very slight though already clearly multilayered. This rudiment, as seen from the reconstruction, is a distinct gland rudiment (gland III) although still of an elongated form. The rudiments soon become thicker and more sharply delimited, as shown in Pl. 1, fig. 3, representing also a section of an 11-day-old embryo. In 12 day-old embryos the epidermal thickening is already transformed into a hemisphere (Pl. 1, fig. 4) sometimes called the 'mammary hillock' and soon after the gland rudiment becomes a spherical body connected with the epidermis only by a narrow neck (Pl. 1, fig. 5). The glands retain this spherical form up to the age of 15 days, and sometimes even to 16 days, but then there begins a new phase in their development: the rudiment begins to elongate and produces the primary sprout (Pl. 1, fig. 6). The single (in the mouse) primary sprout soon begins to produce secondary sprouts, so that by the time of birth (which in my material occurred 19–20 days after fertilization) each gland is represented by a ramifying system of ducts.

DATA ON THE GROWTH AND PROLIFERATION IN THE GLANDS

Table 1 presents a comparison of the growth of the mammary glands with the growth of the whole embryo.

It is evident that for several days after the formation of the mammary gland rudiments their growth lags behind that of the body as a whole. Between the 11th and the 15th day the mammary gland rudiments increase about four and a half times. In the same period the whole embryo increases more than ten-fold. But in the last days of pregnancy the relations are reversed. Between the 16th and the 19th day of development there is at least a four-fold increase in the volume of the mammary

Table 1. *Growth of mammary gland compared with the growth of the whole embryo*

(Mammary glands in arbitrary units (surface of sections), whole embryo in milligrams.)

Age in days	Mammary glands (single observations)	Whole embryo (average)
10	—	11·3
11	530 829 1055	17·95
12	788 828 1791	50·5
13	1027 1804 1944	105·3
14	3548 3680	139·5
15	3354	206·2
16	7497	415·5
17	5559 7806	544·2
18	19020 20950	758·9
19	36174	862·0

Table 2. *The mitotic index of the mammary glands and the epidermis*

Age in days	Epidermis		Mammary glands	
	Counts*	Mitotic index	Counts*	Mitotic index
10	1472 (21)	1·42	—	—
	875 (11)	1·25	—	—
11	510 (4)	0·78	609 (2)	0·32
	507 (2)	0·39	614 (2)	0·32
	772 (7)	0·90	1031 (1)	0·09
12	622 (3)	0·48	940 (3)	0·31
13	915 (8)	0·87	1580 (2)	0·12
14	935 (7)	0·74	2311 (2)	0·08
	1270 (16)	1·26	2068 (6)	0·29

	Proximal part of gland		Distal part of gland		Mitotic index for proximal and distal parts together
	Counts*	Mitotic index	Counts*	Mitotic index	
16	2180 (21)	0·96	2165 (36)	1·66	1·30
	1804 (5)	0·28	1670 (13)	0·78	0·52
17	1935 (21)	1·08	1850 (42)	2·27	1·66
	2649 (21)	0·79	1523 (22)	1·44	1·03
	1424 (12)	0·84	1253 (19)	1·51	1·16
	974 (8)	0·82	1641 (9)	0·55	0·65
	1908 (22)	1·15	1818 (24)	1·32	1·23
19	608 (11)	1·81	1850 (26)	1·40	1·50

* The figures in brackets show the number of mitoses.

gland rudiments, whereas the weight of the whole embryo is only doubled. This change coincides with the beginning of sprouting.

Table 2 presents the data on the mitotic activity in the mammary gland rudiments and in the epidermis.

It will be seen that the mitotic activity shows the same difference between the early (non-sprouting) mammary glands and the late (sprouting) mammary glands, as was found for the increase in volume of the glands. A comparison between the mitotic indices of early and late mammary glands cannot be made by the method of weighted differences (see below), as the data cannot be grouped in pairs; it will be necessary, therefore, to compare the average mitotic indices for both groups in the usual, though not very exact, way.

Average mitotic index for glands of 11- to 14-day-old embryos	$0{\cdot}218 \pm 0{\cdot}043$
Average mitotic index for glands of 16- to 19-day-old embryos	$1{\cdot}132 \pm 0{\cdot}138$
Difference	$0{\cdot}914 \pm 0{\cdot}145$
Ratio: difference/standard error of same	6·30
Considering 13 degrees of freedom	$P < 0{\cdot}001$

The difference is therefore fully significant statistically.

A comparison of the mitotic index of early mammary glands and of the epidermis can be carried through by the method of weighted differences. Table 3 presents the

Table 3. *Comparison of the mitotic indices of mammary glands and of the epidermis in* 11- *to* 14*-day-old embryos by the method of weighted differences*

Epidermis	Mammary gland	Difference	Weight $\left(\frac{N_1 N_2}{N_1 + N_2}\right)$
0·78	0·32	0·46	277·6
0·39	0·32	0·07	277·7
0·90	0·09	0·81	454·0
0·48	0·31	0·17	374·3
0·87	0·12	0·75	579·4
0·74	0·08	0·66	665·7
1·26	0·29	0·97	786·8

Interaction $\chi^2 = 6{\cdot}34$, therefore for 6 degrees of freedom $P = 0{\cdot}30$–$0{\cdot}50$. Weighted difference = 0·6487. Weighted standard error = 0·1185. $t = 5{\cdot}47$, therefore for 6 degrees of freedom $P = 0{\cdot}001$.

respective data. The comparison, carried through by this very strict method, proves that there is a fully significant difference between the mitotic indices of the mammary glands and of the epidermis, from which the mammary glands have been derived. It follows that the formation of the mammary glands is accompanied by a considerable decrease in the mitotic activity of the cells partaking in this morphogenetic process.

The method of weighted differences may be further applied to the comparison of the proximal and distal parts of the glands* in stages when the sprouting has already begun (16- to 19-day-old embryos). The data are presented in Table 4. The calculations support the conclusion that the mitotic activity is higher at the distal ends of the glands, which corresponds to the impression one gets when studying sections of sprouting glands. The degree of significance is in this case very much less than in the case of the comparison of the mitotic indices of mammary glands and epidermis, or of mammary glands of early and late embryos. Still, the probability is well beyond the 5 % margin.

* By 'proximal' is meant the part of the gland connected with the epidermis; by 'distal'—the sprouts growing into the subcutaneous connective tissue.

Table 4. *Comparison of the mitotic index in the proximal and distal parts of sprouting mammary glands by the method of weighted differences*

Proximal parts of glands	Distal parts of glands	Difference	Weight $\left(\frac{N_1 N_2}{N_1 + N_2}\right)$
0·96	1·66	0·70	1086
0·28	0·78	0·50	867
1·09	2·27	1·18	945
0·79	1·44	0·65	967
0·84	1·51	0·67	666
0·82	0·55	−0·27	611
1·15	1·32	0·17	930
1·81	1·40	−0·41	457

Interaction $\chi^2 = 11{\cdot}84$, therefore for 7 degrees of freedom $P = 0{\cdot}10$–$0{\cdot}20$. Weighted difference = 0·489. Weighted standard error = 0·173. $t = 2{\cdot}83$, therefore for 7 degrees of freedom $P = 0{\cdot}02$–$0{\cdot}05$.

CONCLUSIONS

From what has been said it appears that in the embryonic development of the mammary glands three distinct phases may be distinguished, each with its characteristic processes of growth and differentiation.

The first phase is the formation of epithelial thickenings, the mammary buds. During this stage the growth rate of the rudiments decreases from the high level intrinsic for the epidermis to the low level found later in the gland rudiments. This decrease of growth rate may well be the result of a differentiation process taking place in the gland rudiments (compare Schmalhausen, 1927).

There follows a second phase during which the gland rudiments are rather passive in every respect. They show no progressive differentiation and their growth is comparatively small. The glands are negatively heterogonic in respect of the whole embryo. This phase of a relative standstill in development continues from the age of 11 days to the age of 15 or even 16 days.

The third phase is the period of sprouting. The formation and the elongation of the sprouts is due to a growth process and not to a pure change of form and though the growth is particularly rapid at the ends of the sprouts it is not exclusively terminal. The rate of proliferation (mitotic activity) is increased roughly five-fold above the level of the preceding phase. There is a period when the growth activity of the glands exceeds that of the whole embryo—the gland becomes positively heterogonic. This is the more remarkable since after birth the growth of the mammary glands is not positively heterogonic. Cowie & Folley (1949) report that in the rat the growth of the mammary glands is isometric for about 3 weeks after birth, and only then becomes positively heterogonic, in connexion with approaching puberty.

There is good reason for believing that the second phase in the development of the mammary glands—the phase of retarded growth and differentiation—is of widespread occurrence in mammals. In his investigation on the development of the mammary glands of the cow, Turner (1930) states that after the formation of the spherical mammary gland rudiment the same does not increase appreciably in size for about 3 weeks (it is almost the same in 2·4 cm. embryos as in 8·4 cm. embryos). Bresslau (1920) finds that in the Opossum, *Didelphys*, the spherical mammary gland rudiments, although formed in early embryonic stages, persist in this form without

further change until after the marsupial period of postnatal development. I have found the same phase of retarded growth and differentiation in the rabbit, where it continues from the age of 13 days to shortly before the age of 25 days.

SUMMARY

1. The intra-uterine development of the mammary gland rudiments in the mouse was studied by preserving and sectioning embryos of known age. The rate of growth of the mammary gland rudiments was determined by calculating the mitotic index and by measuring the surface of each section drawn with the aid of a camera lucida. The rate of growth of the mammary gland was compared with that of the epidermis and with the growth of the whole embryo.

2. There is no mammary line in the mouse, each gland making its appearance independently of the others. Epidermal thickenings representing individual mammary glands were found in advanced 11-day-old embryos.

3. In newly formed mammary gland rudiments (mammary buds) the rate of growth and cell proliferation decreases far below the level intrinsic for the epidermis from which the mammary gland rudiments have been derived. There follows a period of relatively very slow growth accompanied by an absence of progressive differentiation, except that the original lenticular thickening is transformed into a hemisphere and then into a sphere connected with the epidermis by a narrow neck. In this state the rudiments persist for several days.

4. The phase of arrested development is followed by a phase of more rapid growth and cellular proliferation. This is the period of sprouting when first the primary sprout is formed and then the ramifying duct system of the gland. During this period the growth rate of the gland rises above the growth rate of the whole embryo. Growth is especially rapid in the distal parts of the sprouts although it is not exclusively terminal.

5. It appears that a period of arrested development, with very low rate of growth and absence of changes of form, occurs in the embryonic development of most if not all mammals.

I am greatly indebted to Prof. C. H. Waddington who prompted me to undertake this investigation and who supplied me with the means to carry it through. In the course of the work and during the preparation of the manuscript I have received valuable assistance from a number of persons, Prof. J. C. Brash, Department of Anatomy, University of Edinburgh, Dr D. S. Falconer, Dr M. Latyszewski, Dr E. Reeve and Dr A. Robertson of the Institute of Animal Genetics; Mr T. Janikowski for preparing the sections and to Mr G. Knight for the photographs. To all of them I am very grateful.

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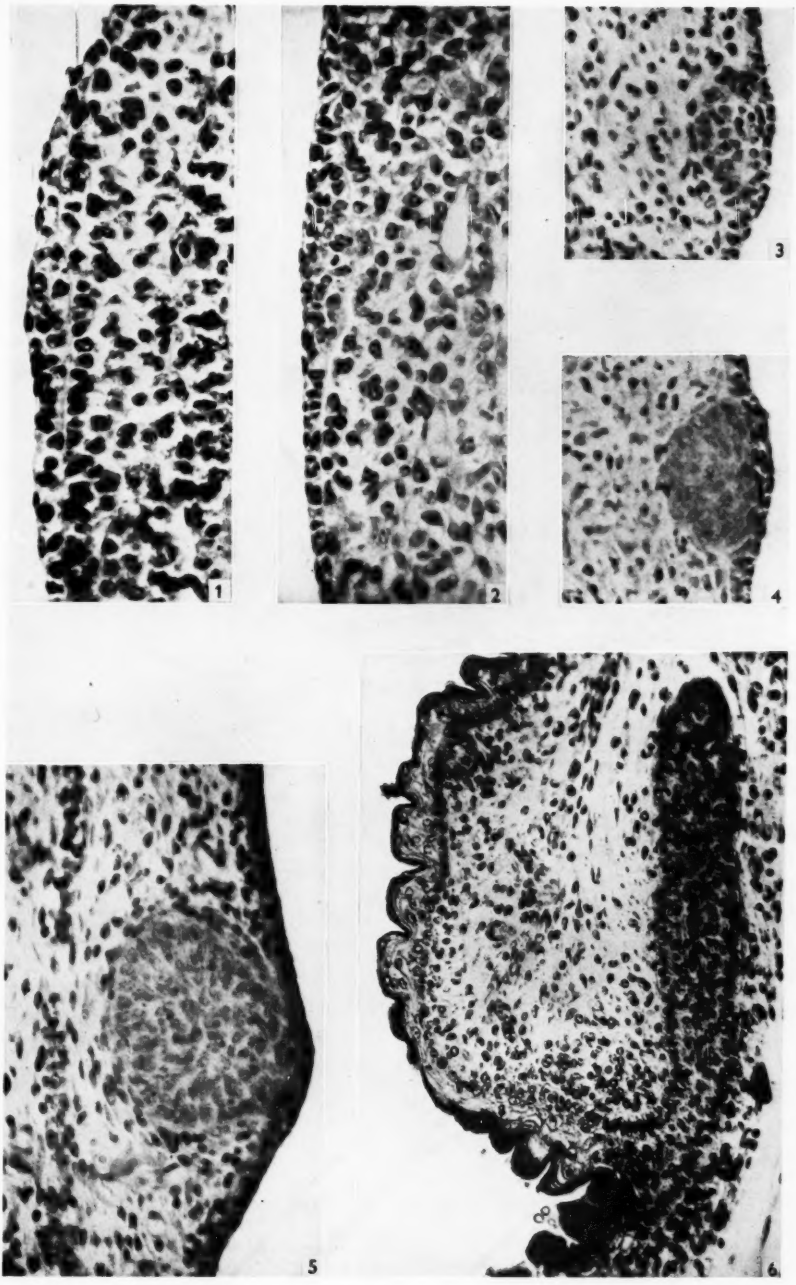

BALINSKY—PRENATAL GROWTH OF MAMMARY GLAND

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EXPLANATION OF PLATE

Fig. 1. Earliest epidermal thickening (rudiment of mammary gland) in an advanced 10-day-old embryo. ×400.

Fig. 2. Epidermal thickening (rudiment of mammary gland) in an 11-day-old embryo. ×400.

Fig. 3. Mammary gland rudiment of an advanced 11-day-old embryo. ×310.

Fig. 4. Mammary gland rudiment (mammary hillock) of a 12-day-old embryo. ×310.

Fig. 5. Fully developed spherical mammary gland rudiment. ×310.

Fig. 6. Mammary gland rudiment of a 17-day-old embryo: the primary sprout. ×200.

COMPARISON OF FRACTURE REPAIR IN THE FROG, LIZARD AND RAT

By J. J. PRITCHARD and A. J. RUZICKA
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INTRODUCTION

Though much work has been done on fracture repair in mammals, it was felt that before any satisfactory general theory of fracture repair could be formulated, much more must be known about the process as it occurs in other vertebrates. It was therefore decided to investigate the healing of fractures in the frog, lizard and rat, with the object of separating general features from those peculiar to the species or class.

Very few previous comparative studies of fracture repair have been made. Fujinami (1901) investigated thirteen species of mammal, bird, reptile and amphibian, but only a few animals were used in each series, and no attempt was made to summarize the results or to deduce general principles from them. Wallis (1928) studied certain aspects of repair in the lizard and rat, but his observations lacked objectivity, as they were used chiefly to support Erdheim's (1914) mechanistic theories. The best and most useful account of repair in Amphibia is that of Wurmbach (1927), who worked with newts, salamanders and frogs.

MATERIALS AND METHODS

Twenty-two frogs (*Rana temporaria*), twenty lizards (*Lacerta vivipara*) and forty rats (*Mus norvegicus*) were used. One femur was fractured in each of the frogs, one femur in thirteen of the lizards and both femora in seven, one femur in ten rats and one tibia in the remaining thirty.

Closed fractures were made under ether anaesthesia by bending the limb sharply against the edge of the operating table. No attempt at immobilization of the fracture was made. The animals showed surprisingly little disability afterwards and were soon moving about almost normally.

The frogs and lizards were fed *ad lib.* on flies and fly larvae. The rats had a generous, well-balanced diet. The frogs were housed in a Perspex tank, in running water, the temperature of which could be regulated by means of a thermostatically controlled heating element. Some of the frogs were kept at room temperature (approx. 18° C.), others at 26° C. It was not possible to keep them alive at temperatures much higher than this. The lizards were kept in small cages of which the internal temperature could be controlled by means of electric light bulbs. They were maintained at temperatures of 18, 26 and 32–37° C., and their activity increased markedly with each rise of temperature.

At selected intervals after the fracture had been made the animals were killed with chloroform, or by decapitation, and the damaged segment of the limb was removed, skinned and placed in either Bouin's fluid or absolute alcohol. The former fixative was used when general staining methods were subsequently to be employed, the

latter when the distribution of inorganic salts and alkaline phosphatase was under investigation. The Bouin-fixed specimens were decalcified in 5 % trichloracetic acid. The others were not decalcified.

The majority of the specimens were embedded in paraffin and cut serially at 7–10 μ. A few of the tibial specimens in the rat were embedded in celloidin and cut at 20 μ. In a few of the rats the blood vessels were injected with indian ink before fixation. No special methods for cutting undecalcified specimens were used. In ordinary paraffin sections it was found that the shaft of the old bone was usually fragmented and displaced but the calcified cartilage and the callus cut fairly easily and their relationships to the soft parts were not seriously disturbed in the majority of sections.

Most of the common staining techniques for bone, cartilage and connective tissue were tried, but those found most generally useful were Weigert's iron haematoxylin and van Gieson, for demonstrating bone matrix and collagenous fibres; and 1 % aqueous methylene blue for cartilage. It was found that these stains could be used in combination, provided that the excess methylene blue fixed to the picric acid of the van Gieson stain was removed with absolute acetone. This combination was most useful for studying the general distribution of bone and cartilage in the same section. For more detailed studies, however, it was found best to use the stains separately on alternate sections. The finest collagenous fibres were not shown by van Gieson's stain, but were sharply defined by Wilder's silver diammino-hydroxide technique.

Inorganic phosphates were demonstrated by converting them to cobalt sulphide. The sections were treated for 5 min. with 2 % cobalt nitrate solution, washed thoroughly in distilled water and then placed in dilute ammonium sulphide solution as in the final stages of the Gomori technique for alkaline phosphatase. Inorganic phosphates were thus stained black. Alkaline phosphatase activity was investigated by Gomori's technique. Usually alternate sections were stained for phosphates and phosphatase.

RESULTS

(1) *General features of the repair process*

In all the experiments, examination of the fracture site 1–2 days after operation showed that the bone had been broken cleanly but that the broken ends lay at an angle to each other and in the case of the fractured femora a certain amount of overlap was also present, but in the tibiae this latter displacement was usually slight.

Extravasated blood lay between and around the broken ends of the bones, within the opened medullary cavities and between the muscle fibres, forming a small haematoma in the immediate vicinity of the fracture. The initial muscle damage was slight and few torn or avulsed fibres were seen.

The periosteum was torn from the bone to a varying, but generally slight extent near the fracture. Periosteal detachment was more marked in the rat than in the other animals, and was greatest on the overlapping surfaces of the fragments. Widespread subperiosteal extravasations of blood were not found.

After the fracture, at times varying with the species and the environmental temperature, but always within a few days, cellular multiplication began around the injured area, first in the *undamaged* periosteum (Pl. 2, fig. 16), then in the inter-

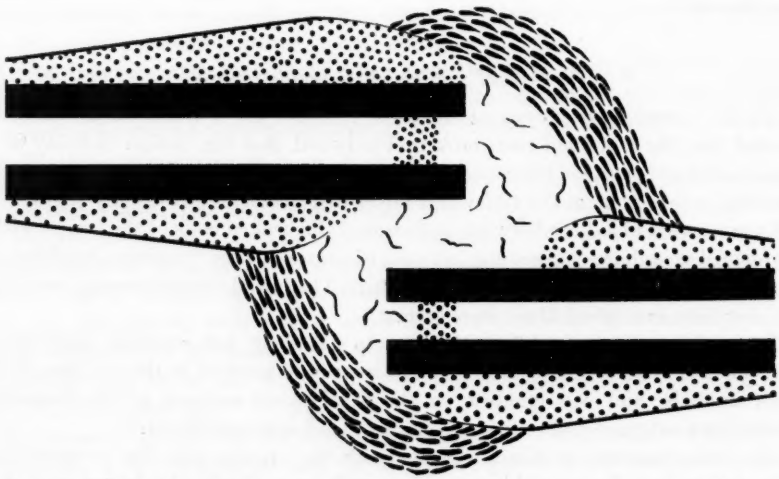
muscular connective tissue and lastly in the medullary cavity. Before long the haematoma was encompassed on all sides by a dense zone of proliferating cells, constituting a regeneration, or reparative, blastema (see Text-fig. 1 and Pl. 1, fig. 1).

The medullary contribution to the blastema, or more shortly, the medullary blastema, was confined to the immediate vicinity of the haematoma. The intermuscular, or parosteal, blastema was more extensive, and involved apparently normal musculature some distance from the fracture, while the periosteal blastema extended for a considerable distance, particularly in the rat, where almost the entire shaft up to the articular extremities was involved. The parosteal blastema was most conspicuous in the rat, less so in the lizard and least in the frog.

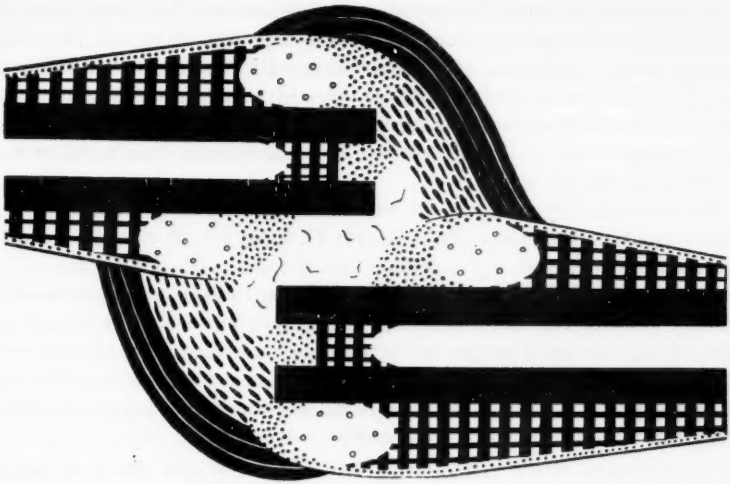
Meanwhile phagocytic cells, polymorphonuclear at first but later almost exclusively mononuclear, invaded the haematoma from around its periphery and the extravasated red blood cells were removed, leaving a network of fibrin strands in place of the original blood clot.

The haematoma was then invaded by cells from the surrounding blastemata. The peripheral part was rapidly replaced by an immature, highly cellular connective tissue, but the central part remained unorganized for a considerable period.

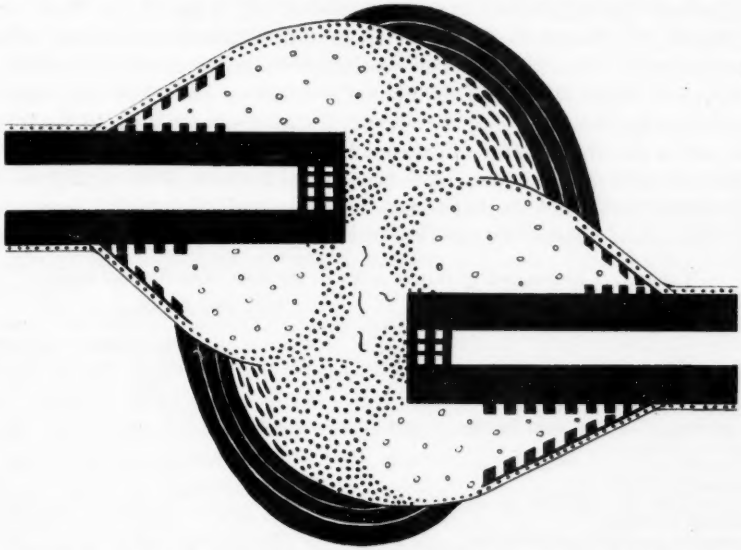
In the frog and lizard the invasion of the haematoma took place predominantly from the periosteal blastema. In the rat parosteal cells were involved to a greater extent, particularly in the overlapping femoral fractures where the majority of the cells replacing the haematoma were derived from the intermuscular connective tissue. The medullary blastema in all species was chiefly concerned in the organization of that part of the haematoma which lay within the medullary cavities, although, at a late stage, it played some part in the organization of the central portion of the haematoma lying between the fragments.



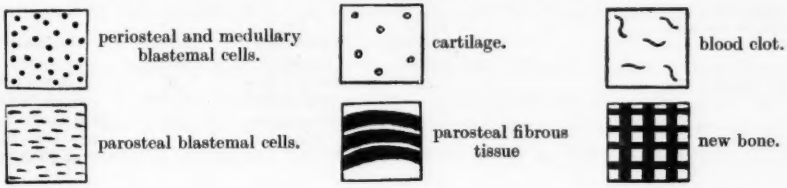
Text-fig. 1. The disposition, in schematic form, of the periosteal, medullary and parosteal blastemata around the fracture haematoma at an early stage. Similar appearances are found in all three species.



Text-fig. 2. The disposition of the new bone, cartilage and fibrous tissue arising in the blastemata of the rat at a later stage than that shown in Text-fig. 1.



Text-fig. 3. A similar stage to that illustrated in Text-fig. 2, but in the frog and lizard.



In the meantime, cellular differentiation and deposition of an intercellular matrix was taking place around the haematoma (see Text-figs. 2, 3). In the thickened periosteum intramembranous ossification occurred in the region remote from the fracture (distal periosteum) while cartilage was laid down near the fracture (proximal periosteum). In the frog and lizard cartilage formation far outweighed that of new bone (Pl. 1, figs. 2, 12); in the rat as much, if not more, bone than cartilage was laid down (Pl. 3, fig. 28; Pl. 4, fig. 29).

Within the medullary cavity, in all species, intramembranous ossification, but not cartilage formation, took place (Pl. 1, fig. 13; Pl. 3, fig. 28). It was also the rule that osteogenesis began later in the medullary cavity than in the periosteum.

The parosteal blastema underwent changes similar to those seen in the maturation of ordinary granulation tissue. The intercellular matrix became increasingly fibrous and this change occurred progressively from without inwards. Bone and cartilage formation did not occur at any stage in the peripheral part of this tissue. The question whether or not the parosteal cells contributed to the formation of cartilage more centrally will be considered at a later stage.

The greater part of the connective tissue which replaced the haematoma was gradually transformed into cartilage, the process beginning as an extension of periosteal chondrification into the fracture gap, and advancing until cartilaginous union between the fragments had been established (Pl. 1, figs. 3, 11; Pl. 2, fig. 22; Pl. 5, figs. 39, 40). Not all the connective tissue differentiated into cartilage, however, for, peripherally, dense fibrous tissue was laid down in the parosteal blastema and this served to repair the gap in the fibrous periosteum, while centrally, strands of fibrous tissue appeared between the advancing masses of cartilage. In the frog and lizard, and in the tibial fractures of the rat this latter formation of fibrous tissue did not prevent cartilaginous union, but in the femoral fractures of the rat fibrous tissue was formed in such amounts that fibrous union resulted. Eventually, however, this fibrous tissue was replaced by cartilage to give a secondary cartilaginous union.

Table 1. *Comparison of repair rates in the four experimental series*

	Days after fracture			
Stages in repair	Rat tibia	Rat femur	Lizard femur (32–37° C.)	Frog femur (26° C.)
Commencement of cellular proliferation in the periosteum	2–3	2–3	4–5	5
First appearance of new bone in the periosteal blastema	3–4	3–4	5	10
First appearance of cartilage in the periosteal blastema	4–5	5	7	10
First appearance of new bone in the medullary blastema	5–7	5–7	7	17
Completion of organization of the haematoma	8	19 (dense fibrous union)	11	17
Commencement of endochondral bone formation	10	10	21	30
Cartilaginous union	10–12 (primary union)	55 (secondary union)	16 (primary union)	36 (primary union)
Bony union	20	Not yet present at 55 days though imminent	31	Not yet present at 70 days. Not much more new bone than at 36 days

The cartilage formed both in the periosteum, and at the site of the original haematoma, was later progressively eroded and replaced by endochondral bone. In the rat and lizard this took place fairly rapidly and led to firm bony union between the fragments (Pl. 1, fig. 5; Pl. 5, fig. 41). In the frog, however, endochondral bone formation was late in starting and progressed at a very slow rate indeed, so that at the conclusion of the experiment (70 days after fracture) most of the cartilage was still uneroded.

(2) *Time-course of repair and the influence of temperature*

The times of appearance of certain critical stages in the repair process in each series are given in Table 1. It will be seen that in the early stages repair in the rat was more rapid than in the lizard which in turn was more rapid than in the frog. The sequence of events, however, was similar in the three species.

In the later stages the repair of the rat's tibia and lizard's femur advanced steadily along parallel courses until bony union had been established on the 20th and 31st days respectively. In the rat's and frog's femora the later stages were greatly delayed, but for different reasons.

In the rat the organization of the haematoma was slow to reach completion, and dense fibrous union resulted instead of the primary cartilaginous union observed in the other cases. This, however, was only a temporary phase, and the fibrous tissue was slowly replaced by cartilage to give a delayed secondary cartilaginous union at the 55th day, when the experiment was completed. Bony union had not then occurred, but appeared to be imminent, for endochondral bone advancing from either side had almost reached the centre of the fracture gap.

In the frog the delay was not in the attainment of cartilaginous union, but in the very slow development of endochondral ossification. When the experiment was completed at the 70th day the greater part of the cartilage originally formed was still present and endochondral bone formation had made little headway in the fracture gap.

In the frog and lizard the rate of repair was markedly influenced by the environmental temperature. In the lizard repair was extremely slow at room temperature (approx. 18° C.), where even after 40 days the centre of the haematoma was still unorganized and no new bone or cartilage had appeared. At 26° C. repair was greatly speeded up, but was still only about half as rapid as at 32–37° C. Thus at 26° C. new bone and cartilage appeared on the 16th day compared with the 5th–7th days at 32–37° C.; and cartilaginous union was incomplete at the 28th day at the lower temperature, although it was already established by the 16th day at the higher. There was little if any difference in the rate of repair in the lizard at 32 and at 37° C.

In the frog repair was considerably faster than in the lizard at room temperature, but at 26° C. this relationship was reversed, in spite of an approximate doubling of the rate in the frog at the higher temperature.

From these results, and from the fact that the lizards remained healthy and active at 37° C. while the frogs were killed by temperatures above 28° C., it would appear that the optimum temperature for repair in the lizard is near the mammalian body temperature, whereas in the frog it is around 26° C. The temperature did not

appear to influence the qualitative aspects of repair, although too few animals were employed at the lower temperatures to establish this with any certainty.

(3) *The periosteal blastema*

In the normal adult frog, lizard and rat, the periosteum consists of a thin layer of mature fibrous tissue containing elongated and flattened cells indistinguishable from ordinary fibrocytes. There is no histological evidence for the existence of a specialized cambial layer of potentially osteogenetic cells beneath the fibrous layer such as is present in the growing animal. For the most part the fibres are orientated parallel to the long axis of the shaft, but some bundles penetrate the shaft obliquely as Sharpey's fibres.

In the early stages of repair the periosteal changes were similar in the three species. They began a few millimetres from the fracture. Mitotic figures appeared and the membrane became progressively thickened, largely as the result of cellular proliferation, but also owing to the occurrence of oedema near the bone. In a short while the thickened periosteum gave the appearance of being divided into an outer and an inner stratum, the former compact and fibrous with fibroblast-like cells between the fibres, and the latter loose and oedematous with ovoid or pyriform cells. A little later the cells nearest the bone became converted into a layer of typical osteoblasts (Pl. 2, figs. 18, 20 and 21). At first sight this appearance suggested that the original periosteum had been passively lifted from the bone by the proliferative activity of a new and independent tissue arising beneath it, and indeed in the literature the formation of the periosteal blastema is often referred to as a 'subperiosteal' reaction. From van Gieson preparations such a conclusion seemed warranted, for the outer layer was chiefly composed of heavily stained, coarse, collagenous fibres similar to those of the resting periosteum, while the inner layer apparently contained few if any collagen fibres. In silver preparations, however, the appearances were very different (Pl. 1, fig. 10), for a rich network of fine fibres showed up in the deeper layer and no sharp dividing line was discernible between the two layers. The fibres merely became finer and more widely separated as one passed inwards, while the original Sharpey's fibres were still present as frayed strands crossing the deeper layer from the outer surface and penetrating the cortex of the bone. While it appeared probable that some of the deeper fibres had been newly formed, the majority had evidently been derived from the stretching and fraying of fibres originally present in the resting periosteum, owing to the accumulation of cells and oedema-fluid between them. The appearances were inconsistent with the view that the original fibrous periosteum had been lifted bodily from the surface of the bone.

There was also no sharp boundary between the cells of the inner and outer strata. The superficially situated fibroblast-like cells, the deeper rounded cells and the definitive osteoblasts lying against the bone formed in reality a continuous series in respect of shape, size, nuclear staining and cytoplasmic basophilia (Pl. 2, fig. 18). Moreover, mitotic figures were most frequent in the outer layer which would not have been the case if this played a passive role while the deeper-lying cells were proliferating. It was therefore concluded that the cells of the periosteal reaction are of similar lineage, the outer layer constituting a reserve of proliferating cells from which

the inner layer of differentiating cells is progressively supplemented. The gradual transition from younger and less differentiated cells in the outer layer to older and more highly differentiated cells in the inner layer naturally follows.

This blastemal reaction was at first confined to the undamaged periosteum a little distance from the fracture; no cellular proliferation was observed at any stage in the avulsed periosteum in the immediate vicinity of the haematoma. As the reaction developed the periosteum was involved at increasing distances from the fracture. The thickened periosteum became wedge-shaped in section, being thickest proximally, i.e. towards the fracture, and tapering distally into normal periosteum away from the fracture. In the rat the transition from thickened to normal periosteum was gradual so that the wedge had a narrow angle, but in the frog and lizard the change was more abrupt, and took place relatively nearer the fracture than in the rat, so that the wedge was of wider angle (c.f. Text-figs. 2, 3 and Pl. 1, fig. 2, with Pl. 3, fig. 28).

In all three species the single layer of osteoblasts lying against the shaft became associated early on with the deposition of a thin lamella of new bone between it and the original bone. In the rat this was rapidly followed by extensive intramembranous ossification throughout the deeper layers of the periosteum in the distal part of the wedge (Pl. 3, fig. 28; Pl. 4, fig. 29), the process being heralded by the differentiation of the periosteal cells into typical osteoblasts. Later the cells in the thick part of the wedge near the fracture differentiated into chondroblasts and cartilage was laid down (Pl. 4, fig. 32; Pl. 5, fig. 39).

In the frog and lizard this extensive formation of new bone in the distal periosteum did not occur, and almost the whole of the periosteal blastema underwent chondrification (Pl. 1, fig. 3). In all species, however, a layer of proliferating cells remained just beneath the fibrous outer layer of the periosteum external to the new bone and cartilage. From these cells were differentiated successive layers of either osteoblasts or chondroblasts which contributed to the increasing thickness of the bone and cartilage. Eventually the new formation of chondroblasts ceased and a thin layer of new bone was deposited between the cartilage and the fibrous periosteum. This outer bony shell was particularly conspicuous in the later stages of repair in the frog and lizard, where it gradually spread towards the fracture (Pl. 1, fig. 3) and eventually, in the lizard, provided the chief bond uniting the fragments after the cartilage had been resorbed (Pl. 1, fig. 5).

With the cessation of osteogenesis and chondrification in the periosteum the outer fibrous layer became the new periosteum. This did not involve any loss of continuity between the various tissues, for in silver preparations it was seen that the fibrous bases of the cartilaginous and bony matrices were continuous both with one another, and with the fibrous tissue of the new periosteum.

There were marked differences in the vascularity of the periosteal blastema in the three species, and these appeared to have an important bearing on the type of matrix deposited. In the rat the distal part of the periosteal blastema was very vascular and the bone deposited here showed an alternation of blood vessels and bony trabeculae similar to that seen in the course of normal intramembranous ossification. The proximal part of the blastema, however, where chondrification took place, was almost entirely avascular.

The transition from typical eosinophil trabecular bone to typical basophil cartilage as one passed towards the fracture line was not abrupt in the rat, for between them there was differentiated a zone of connective tissue having properties intermediate between those of bone and cartilage, and unlike anything seen in the course of normal embryonic development (Pl. 4, fig. 32). The bony trabeculae first became coarser, more cellular and less eosinophil as the blood vessels became less numerous; then the trabecular structure was lost and the cells became intermediate in type between those of cartilage and bone, while the matrix stained faintly with both acid and basic dyes; then some of the cells acquired faintly basophil capsules and finally the tissue merged into frank avascular basophil cartilage.

In the lizard and frog the vascular pattern was quite different. In spite of prolonged search only an occasional blood vessel could be found in the thickened periosteum of the lizard and, in the majority of sections, the tissue appeared to be entirely avascular. In the frog a few blood vessels, widely spaced, were present in the blastema before chondrification began. Later these came to lie within a sparse system of cartilage canals (Pl. 2, fig. 19), where, in some places, a thin layer of bone was deposited around the vessels against the cartilage.

The formation of cartilage in the periosteum was thus associated in all three species with poverty of blood supply, while, on the other hand, bone formation was limited to the neighbourhood of blood vessels. Thus in the rat the extensive formation of new bone in the distal part of the periosteum was associated with an equally extensive system of blood vessels, while nearer the fracture the formation of cartilage took place in an avascular environment. Between the two, as the vascularity diminished, bone merged into cartilage through a series of intermediate stages.

In the lizard and frog the blood supply was poor throughout the blastema and cartilage was formed predominantly. What little new bone was present in the early stages of repair lay either close to the original shaft, where it was near cortical blood vessels; or immediately beneath the fibrous periosteum, where it was not too remote from the blood vessels of the extraperiosteal connective tissue; or on the walls of vascular cartilage canals.

The transition from bone to cartilage in the frog and lizard was more abrupt than in the rat, but on close examination a layer of an intermediate type of tissue could always be distinguished between them.

The modes of differentiation of true bone and cartilage within the periosteal blastema were essentially similar to those seen in the course of normal osteogenesis and chondrogenesis in embryonic life. The early blastema, in its deeper layer, consisted of a relatively homogeneous mass of cells (Pl. 2, figs. 18, 21) embedded in a matrix of fine collagenous fibres. The cells were generally ovoid with a centrally placed, moderately chromatic nucleus and a juxta-nuclear vacuole of moderate size, while the cytoplasm showed a definite affinity for such dyes as thionin, methylene blue and pyronin.

Where bone was to be formed the cells became pyriform with an eccentrically placed, heavily staining nucleus, the juxta-nuclear vacuole increased markedly in size and the cytoplasmic basophilia became greatly intensified. The cells now had the typical appearance of osteoblasts. Between them the collagen fibres became aggregated into coarse strands and a homogeneous cementing substance which

stained intensely with acid dyes was deposited around them. The fibres now were masked and could no longer be seen in van Gieson preparations, though they could still be seen clearly after silver impregnation. At this stage the tissue was readily identifiable as bone matrix, but, as will be shown later, no bone salts had yet been deposited.

The thin trabeculae of new bone bore a constant relationship to the vascular network, lying equidistant from neighbouring vessels, and the osteoblasts became arranged as a pseudo-epithelial surface layer. This early bone therefore had the appearance of typical primary cancellous bone. Later, in some situations, lamellar bone was deposited on the surfaces of the trabeculae, which were correspondingly thickened; in other situations osteoclasts appeared and the primary trabeculae were removed, opening up wide spaces which became filled with haemopoietic marrow (Pl. 5, fig. 41).

Where cartilage was to appear the cells became larger and more nearly spherical and their nuclei stained less heavily with haematoxylin while the cytoplasmic basophilia gradually decreased in intensity. A basophil matrix was then deposited around the cells in the form of thin capsules. At first the cells with their capsules were separated by tracts of fine collagen fibres, but as the deposition of the matrix spread away from the cells these were engulfed and were then no longer visible after ordinary staining methods, though, as in the case of bone, they were still visible in silver preparations.

In the rat and lizard the cartilage formed in this way showed a high ratio of cells to matrix and this hypertrophic condition persisted. In the frog, however, the proportion of matrix gradually increased until the cartilage was of the hyaline variety such as is normally found in the epiphyses of this animal.

Between the areas of normal bone- and cartilage-formation the cells were predominantly large and rounded as in cartilage, but they showed the large juxta-nuclear vacuoles and intense cytoplasmic basophilia typical of osteoblasts. The intercellular matrix was of heterogeneous consistency, with a tendency towards the appearance of thin basophil capsules around the larger cells and an eosinophil matrix elsewhere. The original collagen fibres were incompletely masked by the matrix and many of them remained visible in van Gieson preparations.

(4) *The medullary blastema*

The development of the medullary blastema proceeded along similar lines in the three species. The normal marrow structure disappeared near the haematoma and was replaced by a mass of closely packed proliferating cells of the same general type as those present in the early periosteal blastema. From their first appearance in all species the blastemal cells were associated with a rich network of blood vessels. The origin of the cells remained in doubt, but most probably they were derived from the reticular cells of the marrow. In the normal bone there was no sign of any cambial layer of potentially osteogenetic cells lining the interior of the shaft.

The blastemal cells gradually replaced the haematoma within the marrow cavity up to the fracture line, and also for a short distance into the fracture gap. Their advance was slower than that of the periosteal and parosteal cells, so that they

played a relatively small part in the repair as a whole. The cells nearest the inner aspect of the bone soon differentiated into osteoblasts, and trabeculae of new bone were laid down first of all in their vicinity (Pl. 1, fig. 13). Before long the majority of the cells followed suit and a network of bony trabeculae was formed throughout the blastema (Pl. 3, fig. 28).

The chief result of the activity of the medullary blastema was the formation of a plug of membrane bone sealing off the medullary cavity at the fracture line. This played very little part in effecting bony union between the fragments except in the tibial fractures of the rat where the medullary cavities were in close apposition, and a bridge of new bone was erected between them in the later stages of repair after the medullary blastemata had met across the fracture gap (Pl. 5, fig. 41).

In view of what has already been said about the relationship between vascularity and osteogenesis, it was noteworthy that the medullary blastema, which was always well supplied with blood vessels, did not show cartilage formation in any species.

(5) *The parosteal blastema*

The changes which took place between the muscle fibres near the fracture were similar to those seen around any inflammatory focus. The connective tissue cells proliferated and a wall of young granulation tissue was erected around the haematoma (Pl. 3, fig. 28). During this process many of the muscle fibres included between the strands of proliferating connective tissue became fragmented, while others apparently disappeared. At a later stage, however, regeneration cones were seen sprouting from the ends of many of the fragmented fibres and eventually a more or less complete restoration of the muscles was effected. This observation confirms Murray & Kodicek's (1949) results in experimental fractures of the fibula in guinea-pigs.

As the granulation tissue matured it became increasingly fibrous and more and more restricted to the periphery of the haematoma, while at the sides the line of demarcation between it and the fibrous periosteum was gradually lost. The parosteal blastema thus appeared to be instrumental in restoring the continuity of the fibrous periosteum across the fracture gap. Beneath this fibrous stratum, however, some parosteal cells invaded the haematoma, but discussion of their role in the repair process will be deferred until the organization of the haematoma is dealt with as a whole in the next section.

(6) *The organization of the haematoma*

The haematoma was invaded around its periphery by cells derived from the periosteal, parosteal and medullary blastemata (Text-figs. 1–3). At first the separate invasion paths were clearly distinguishable for they were limited by such natural topographical features as the fibrous outer layer of the periosteum, and the shaft of the fractured bone. As the cells encroached on the more central parts of the haematoma, however, it became increasingly difficult to distinguish them from one another by situation alone and in particular, to distinguish periosteal cells from those of parosteal origin.

There were other criteria by which the cells could be identified, but these became

increasingly difficult to apply as the centre of the haematoma was approached. Thus the periosteal cells, in general, were larger, more closely packed and showed more intense cytoplasmic basophilia than the parosteal cells. The reaction for alkaline phosphatase, however, provided the surest method of distinguishing the cells, for those from the periosteum exhibited intense enzyme activity while the parosteal cells showed little or none. Unfortunately this test could not always be applied for, owing to difficulties inherent in cutting undecalcified material, the most important parts of the sections were often lost.

In spite of these handicaps there was little doubt that in the frog and lizard and in the tibial fractures in the rat, the periosteal cells played the chief role in the organization of the haematoma, while the role of the parosteal cells was largely restricted to the restoration of the fibrous periosteum. On the other hand, in the femoral fractures in the rat, the haematoma was organized very largely by cells of parosteal origin.

To begin with, the haematoma was replaced by a primitive connective tissue containing fusiform cells and a network of very fine collagenous fibres, the majority of which could only be shown after silver impregnation. There were a few blood vessels in the rat and frog, but none in the lizard.

In the frog and lizard and tibial series in the rat the greater part of the primitive connective tissue became directly transformed into cartilage. This was not a localized process, for chondrification advanced in an orderly manner into the fracture gap from the periosteum on either side, and there was direct continuity between the periosteal cartilage and that formed in the gap (Pl. 1, figs. 2, 3). The cartilage also presented similar histological features in both situations. In the frog the ratio of matrix to cells was everywhere high; in the lizard and rat, low.

Few blood vessels accompanied the blastemal cells into the haematoma. In the frog there were a few vessels of capillary size and these later occupied a sparse system of cartilage canals. In the lizard the cartilage was quite avascular. In the rat the blood vessels became surrounded by sheaths of fibrous tissue and these, intersecting the cartilage, gave it a somewhat lobulated appearance.

In all species there was a tendency for the cartilage to degenerate in a few situations. The cells became pyknotic and the matrix was resorbed leaving cyst-like spaces (Pl. 2, fig. 22).

In the femoral series in the rat the conditions were very different. The spread of chondrification into the fracture gap from the periosteum made little headway, and the bulk of the primitive connective tissue became transformed into dense, almost avascular, fibrous tissue interspersed with slit-like cavities resembling bursae. Later, when the periosteal cartilage had all been replaced by endochondral bone, chondrification began anew in the fibrous tissue (Pl. 4, figs. 30, 33), and this led eventually to a secondary cartilaginous union. The secondary cartilage had a different structure from that of the primary cartilage laid down in the periosteum. The latter was of the hyaline variety in which the fibres were masked while the former had the appearance of fibro-cartilage, for the individual cells and their basophil capsules were separated by strands of mature fibrous tissue which stained intensely with van Gieson. The significance of this appearance of two types of cartilage in the course of fracture repair will be discussed later.

(7) *Replacement of cartilage by bone*

The most striking differences between the species in their modes of fracture repair concerned the manner in which the cartilage was resorbed and replaced by endochondral bone.

In the rat resorption began at the junction of the periosteal new bone with the cartilage on either side and advanced towards the centre of the fracture gap (Pl. 5, fig. 40). The invasion front consisted of an advancing line of numerous narrow, closely spaced and parallel erosion bays each of which was occupied by a capillary loop surrounded by blastemal cells. Chondroclasts were present in many of the bays just ahead of the capillary loops. The uneroded cartilage partitions between the erosion bays, left in the rear of the invasion front, became covered with a palisade of osteoblasts and new bone was deposited around them. The resulting trabeculae of endochondral bone retained their cartilaginous cores for some time and the cartilage matrix preserved its ordinary intense basophilia. Endochondral bone formation in the fracture cartilage thus followed very closely the events in the normal epiphysis. The chief difference was that whereas the bulk of normal epiphyseal cartilage is of the small-celled variety and only shows hypertrophic changes in a band just ahead of the invasion front, the fracture cartilage was hypertrophic from the beginning and no further hypertrophy took place.

In the lizard erosion began under the subperiosteal shell of new bone which had previously been formed across the fracture gap (Pl. 1, figs. 4, 7) and not, as in the rat, along linear invasion fronts on each side. Resorption was also a much less orderly process than in the rat and the erosion bays were relatively wide and shallow and irregularly spaced. Each bay was occupied by a centrally placed blood vessel surrounded by blastemal cells, and one or more chondroclasts were usually present. In the rat deposition of endochondral bone followed promptly upon erosion but in the lizard bone formation was delayed. Bone eventually appeared in the form of thin lamellae applied to the surfaces of the uneroded cartilage between the erosion bays (Pl. 2, fig. 17). This residual cartilage formed a network of trabeculae which were much thicker than those of the rat owing to the wider spacing of the erosion bays.

So far, endochondral bone formation at the fracture site in the lizard followed closely that seen at the normal epiphysis in this animal, and the differences between the lizard and the rat at the fracture were paralleled by similar differences at the epiphyseal line; but, in addition, the fracture cartilage in the lizard showed conspicuous changes which were demonstrable only with difficulty at the normal epiphyseal line, and which did not occur at all in the rat. Around the erosion bays the cartilage matrix lost its basophilia and became eosinophil (Pl. 2, fig. 14), and strands of coarse collagenous fibres appeared in it which were easily visible after van Gieson staining (Pl. 2, fig. 15). Moreover, the cells shrank and became more heavily stained until eventually they were indistinguishable from osteocytes. During this process a series of intermediate stages could be identified in a given section between normal basophil cartilage at a distance from blood vessels, and the bone-like tissue described, which lay nearest to blood vessels.

When these appearances were first seen it was thought that possibly the transitional

forms had been present from the beginning, or that the changes were associated with endochondral bone formation in erosion cavities which were sectioned tangentially, but after careful comparison of early with later stages, and of serial sections in the later stages, both these explanations were found to be false. Thus the cartilage before erosion began was homogeneous in its structure and staining properties (Pl. 1, figs. 3, 11), while at its junction with bone only a very narrow band of an intermediate type of tissue was with difficulty distinguishable, whereas after erosion began the intermediate forms were present over wide areas. Then, in serial sections, it was established that the changes were taking place well ahead of the erosion bays in regions where blood vessels and osteoblasts had not yet penetrated. Moreover, the bone laid down on the walls of the erosion bays was of the lamellar type and was covered with a layer of typical osteoblasts, whereas the tissue described above had the honeycomb structure of cartilage although its staining reactions were those of bone and typical osteoblasts were not present (Pl. 2, fig. 17). We were therefore forced to conclude that an actual transformation of cartilage into bone was taking place, involving resorption of the basophil cartilage matrix, substitution of a fibrillar eosinophil matrix, and conversion of the chondrocytes into osteocytes.

In the frog the pattern of endochondral ossification in some respects was like that in the lizard, in other respects it was quite different.

Thus the periosteal cartilage surrounding the ends of the original fragments was eroded in an irregular manner from its external and internal aspects, and wide erosion bays were formed on whose walls lamellae of endochondral bone were deposited, as in the lizard (Pl. 3, figs. 23, 26 and 27). Chondroclasts, however, were very rarely seen. The cartilage was eventually divided up into irregular nodules covered externally by new bone. During the erosion similar changes were observed in the cartilage to those described in the lizard, including loss of basophilia, acquisition of eosinophilia, fibrosis of the matrix and transformation of the cells into osteocytes.

In the cartilage occupying the fracture gap, conditions were quite different. The changes were restricted here to the cartilage canals. Previously these were quite small and were surrounded by homogeneous basophil hyaline cartilage (Pl. 2, fig. 19). Now they became greatly enlarged by the resorption of the cartilage, widening of the blood vessels and increase in the number of the perivascular cells (Pl. 3, fig. 24). During this process some adjacent canals coalesced. The resorption was accompanied by loss of basophilia and acquisition of eosinophilia on the part of the cartilage matrix surrounding the canals. Fibrosis of the matrix, as in the lizard, and in the periosteal cartilage of the frog, was not observed around the canals, and the cells showed little change. The mechanism of erosion was not clear for no chondroclasts were to be seen in any of the canals. The intracanalicular cells nearest the cartilage then became differentiated into osteoblasts, and lamellar bone was formed between them and the cartilage (Pl. 3, fig. 25).

These changes took place very slowly. They first appeared in the canals nearest the ends of the bone on the 30th day, but had not reached the canals at the centre of the fracture gap even after 70 days.

In certain respects the erosion of the fracture cartilage in the frog was similar to that taking place in the epiphyses of the long bones during normal growth. At the junction of the bone marrow with the normal epiphyseal cartilage an invasion front

is normally present where vascular loops and medullary cells advance into wide, shallow erosion bays. No chondroclasts are to be seen and the cartilage is not replaced by endochondral bone. Very significantly, in view of what has been described at the fracture site, the epiphyseal cartilage nearest the marrow cavity is strongly eosinophil in contrast with the greater part of the epiphysis which is strongly basophil, although architecturally the two forms of cartilage appear to be identical. Also the transition from basophilia to eosinophilia is not abrupt but gradually takes place over a fairly wide intermediate zone.

Although evidence of direct transformation of cartilage into bone in the frog could not be found in the epiphyses of the long bones, in the pelvis, where endochondral bone formation does take place, indications of such a process were seen in the cartilage lying between the erosion bays, but on a much smaller scale than that seen at the fracture site.

After a time, in the rat and lizard, the fracture cartilage was entirely replaced by a network of endochondral trabeculae, fine meshed in the rat and coarse meshed in the lizard. The trabeculae in the rat were narrow and consisted of an outer layer of lamellar bone and an inner core of basophil cartilage. In the lizard the trabeculae were much wider and the inner core consisted of a cellular type of bone in which the original cartilage structure, but not its staining properties, was preserved. Later most of the endochondral bone in both lizard and rat was removed in the course of remodelling and replaced by haemopoietic marrow.

In the frog the structure of the endochondral bone was much more like that of the lizard than of the rat. It differed from the lizard chiefly in being more compact.

(8) *Calcification*

Strictly speaking, the available histochemical tests for the mineral salts of bone and cartilage are methods for detecting the presence of insoluble inorganic phosphates. There can be little doubt, however, that these consist predominantly of salts of calcium. By the method used in the present investigation the sites of deposition of these salts are inferred from the locations of deposits of cobalt sulphide in the sections. In order to avoid unnecessary circumlocution the process of deposition of the mineral salts will be referred to as 'calcification' and the salts themselves as 'calcium salts', although the evidence is necessarily circumstantial.

It was found in the case of bone that the intensity of the cobalt sulphide reaction was inversely proportional to the age of the tissue, suggesting that cobalt is able to replace the calcium of recently deposited bone salt relatively easily, but can do so only with difficulty later on. This finding is in agreement with the prevalent view that newly formed bone salt is composed very largely of readily hydrolysable calcium phosphate, but that later this is converted into a complex carbonoapatite in which the calcium is less labile. In cartilage, however, the age of the tissue apparently made no difference to the ease with which its mineral salts were demonstrable.

This ability of the present method to differentiate between recent and older deposits of bone salt is fortunate, for we were naturally most interested in the initial phases of calcification where the reaction was most intense. Another finding was that, whereas in newly formed bone the precipitate of cobalt sulphide was uniformly dense and amorphous, in cartilage it was coarsely crystalline and the overall density

was much less. The reasons for these differences were not clear, but it is tempting to relate them to differences in the fine structure of the organic matrices of the two tissues.

In all three species there was some delay in the calcification of the newly deposited bone matrix. Thus in the trabeculae of membrane bone formed in the periosteal blastema of the rat, the matrix farthest from the old bone, which had been most recently deposited, showed no precipitate of cobalt sulphide (Pl. 5, fig. 35). In the lizard, likewise, the new bone deposited during endochondral ossification at first gave no reaction for calcium salts (Pl. 2, fig. 17). In the frog also an uncalcified layer of bone matrix was found between the calcified bone matrix and the osteoblasts during endochondral bone formation in the walls of the cartilage canals (Pl. 3, fig. 25).

In the case of bone matrix, while the evidence for delay in calcification was quite definite, the actual time lag was probably quite short, and at most was only of the order of a day or two. In the case of cartilage, however, the delay was much greater.

Thus, in the lizard, cartilage appeared in the periosteal blastema on the 7th day, but calcium salts were not deposited until the 16th day, by which time cartilaginous union had been effected. Even then, only the oldest parts of the cartilage farthest from the fracture showed deposits of calcium salts, which were present as coarse crystals in the capsules of the more peripherally placed cells (Pl. 1, fig. 9). From here calcification slowly spread towards the fracture. It did not take place indiscriminately throughout the thickness of the cartilage but only under the fibrous periosteum and on the surface of the old bone. Then as erosion began calcium salts were deposited just in advance of the irrupting blood vessels in the zone where direct transformation of bone into cartilage was taking place. This relationship of calcification to the advance of blood vessels was very definite and probably means that the blood is the source of the mineral salts necessary for calcification. In newly formed endochondral bone the cores of the trabeculae, composed of cartilage which had been transformed into bone, were heavily calcified, but the lamellar bone at the surface of the trabeculae was uncalcified (Pl. 2, fig. 17).

In the rat, cartilage first appeared in the periosteal blastema on the 4th or 5th day, but calcium salts were not in evidence until the 12th day, and even then they were only present in the neighbourhood of the blood vessels (Pl. 5, fig. 37). As in the lizard, this restriction of calcification to the neighbourhood of blood vessels persisted throughout endochondral ossification.

In the frog, that part of the cartilage which was farthest from the fracture became heavily calcified as it was being transformed into bone, but the bulk of the cartilage was completely devoid of calcium salts except for a short distance around the cartilage canals, and even there they were not always present (Pl. 3, fig. 25).

(9) *Phosphatase activity*

In the rat and lizard the osteogenetic and chondrogenetic tissues around the fracture site always gave a strong reaction for the enzyme after a short period of incubation. In the frog, however, only a faint reaction could be elicited even after prolonged incubation, although the specimens from all species were treated similarly prior to incubation. The normal epiphyseal cartilage in the frog, unlike that of the

lizard and rat, also exhibited little or no enzyme activity. The reason for this difference between the species is not known, but possibly the enzyme in the frog is particularly thermo-labile and is almost entirely destroyed in the paraffin oven. On the other hand, it may be that phosphatase activity is in fact very low in the frog and this may be an explanation of the virtual absence of calcification in the fracture cartilage. The observations which follow will therefore be confined to the rat and lizard. In both animals the periosteal and medullary blastemata gave strong reactions, but the parosteal cells showed little or no enzyme activity (Pl. 1, fig. 6; Pl. 4, fig. 31; Pl. 5, fig. 38).

The enzyme appeared as soon as the periosteal and medullary cells began to proliferate, and before bone or cartilage had been formed or calcium salts deposited. At this stage the nuclei, especially the nuclear membranes and nucleoli, and the intercellular collagen fibres gave the strongest reaction, although the cytoplasm generally showed some activity. In the periosteum all the cells, whether of the outer or inner layers, gave the reaction. As bone and cartilage were differentiated the intensity of the reaction waned somewhat in the neighbourhood of the newly deposited matrices, but remained intense in the external zone of proliferating cells. In the region of new bone formation the osteoblasts showed moderately active nuclei and in many cases the Golgi zone was the most active part of the cytoplasm. In the cartilage the activity varied in a patchy manner, some cells showing almost no activity, others having activity concentrated in the capsules, while in other cases the matrix was uniformly and intensely active between the cells. Taken as a whole, however, the concentration of activity in the capsules was the most characteristic feature (Pl. 1, fig. 8; Pl. 5, fig. 36), and it is perhaps significant that the calcium salts deposited at a later stage, had a similar distribution (cf. Pl. 1, fig. 9).

The most striking, and theoretically the most significant, appearances were given at the junction of the periosteal and parosteal blastemata within the fracture gap (Pl. 4, fig. 34). Here the limit of periosteal invasion was marked by an abrupt change from intensely active to almost inactive cells, which paralleled an equally abrupt but less conspicuous change from larger, more closely packed and highly basophil cells to smaller, more loosely packed and less basophil ones. In view of these differences it is difficult to maintain, as Leriche and Policard (1928) and others have done, that the periosteal and parosteal cells are morphologically and functionally equivalent.

In view of the generally accepted thesis that phosphatase activity is an essential accompaniment of normal calcification it was of interest to observe that the appearance of enzyme activity preceded that of calcium salt deposition by a considerable interval. In Pl. 5, figs. 35 and 38, two adjacent sections are illustrated, the former stained for preformed calcium salts, the latter for alkaline phosphatase. It is evident that enzyme activity is well in advance of calcification. In cartilage the interval is much longer, for phosphatase activity is intense throughout the cartilage at the time of cartilaginous union, yet calcium salts are restricted to the peripheral parts near blood vessels. It appears, therefore, that the enzymic capacity for calcification runs ahead of its performance; and also that the presence of the enzyme does not in itself ensure calcification. If, however, it is postulated that the organic phosphates which form the substrate for the enzyme are derived from the blood stream, and do not

diffuse very far into the cartilage, then the restriction of calcification to the neighbourhood of blood vessels would be expected.

In the frog, what little enzyme activity could be demonstrated was limited to the nuclei of the proliferating osteoblasts and the cartilage cells in the immediate neighbourhood of the canals.

CONCLUSIONS AND DISCUSSION

This investigation has shown that, in spite of a number of important differences between the species, and even between the individual specimens from a given species, there exists a fundamental pattern of growth and differentiation during fracture repair which is independent of species and of the type of fracture.

This fundamental pattern seems to involve the following general principles: (1) that repair is due to the combined activities of three blastemal tissues which arise from undamaged periosteal, medullary and intermuscular (parosteal) connective tissues around the fracture site; (2) that the repair process is primarily directed towards the organization of the fracture haematoma from without inwards; (3) that the periosteum possesses a latent, easily-evoked capacity for bone and cartilage production and the medullary tissue for bone production, but the parosteal tissue is primarily fibrogenic although it may give rise to cartilage in certain circumstances; (4) that similar cells are responsible for both bone and cartilage formation in the periosteal blastema; and (5) that the pattern of cellular differentiation and matrix deposition is causally related to the richness or otherwise of the blood supply, and that where abnormal modes of growth and differentiation occur, abnormal conditions of vascularity may be held responsible.

The observations and arguments from which these principles have been derived have already been presented in the earlier part of this paper. A few points, however, need further elaboration, in particular the circumstances in which cartilage is formed, and the role of vascular supply in determining the course of repair.

There was no doubt in our experiments that the bulk of the cartilage arose in the periosteal blastema. The only unequivocal evidence of its formation elsewhere was in the femoral fractures of the rat where it arose in part of the parosteal connective tissue. The deposition of cartilage in the periosteum may seem surprising at first in view of its absence from this situation during normal growth, but the evidence is incontrovertible (see Ham, 1930). The most direct evidence that the periosteum possesses the latent capacity for cartilage production is given by the appearance of both bone and cartilage in cultured explants of periosteum (Fell, 1932, 1933). In bone sarcomata also the periosteum appears to give rise to both types of tissue. Then it must be remembered that in embryonic life the periosteum is derived from the perichondrium of the cartilaginous precursors of the long bones, while throughout the growing period, in the region of the epiphyseal line, periosteum and perichondrium are not sharply distinguishable. It is generally concluded from this evidence that the adult periosteal cells are potentially both osteogenetic and chondrogenetic, and that similar cells may give rise to either osteoblasts or chondroblasts, depending on circumstances external to the cell. Enneking (1948), however, has recently concluded from fracture studies that cartilage and bone are formed from different parts of the periosteum, the former from a specialized cellular cambial layer lying deeply

near the bone, the latter from the fibrous outer layer, but the evidence he adduces is not convincing and is refuted by the occurrence of intermediate types of tissues in which the structure and staining properties of the two tissues overlap.

A more important question is why cartilage is formed in certain situations and bone in others. The evidence has already been presented in favour of the view that bone is formed where the blood supply is rich and cartilage where it is absent or poor. This relationship has been noted by a number of previous workers, and its importance has been stressed by Blaisdell & Cowan (1926) and Ham (1930).

Other workers follow Roux (1895) in emphasizing the role of mechanical factors in determining the course of differentiation, cartilage appearing where the blastema is subjected to pressure and shearing stresses, and bone where the blastema is relatively unstressed. There is much to be said for this point of view, for the deposition of large amounts of cartilage in displaced and poorly immobilized fractures is a common clinical and experimental finding (Rigal & Vignal, 1881; Groves, 1914). Again, Bast, Sullivan & Geist (1925) found that cartilage was formed in much greater amounts in complete than in incomplete saw-cut fractures, while Kapsammer (1898), Wurmbach (1927) and Wallis (1928) agree that it appears in greatest amount on the concave aspect of angulated fractures, where the mechanical stresses are presumably greatest. Conversely, Pritchard (1946) found that cartilage rarely appeared in experimental fractures of the parietal bone where movement and pressure between the fragments is minimal.

In our present experiments, while it is true that cartilage was characteristically formed in the immediate neighbourhood of the ends of the fragments, at first sight supporting the mechanical hypothesis, the constancy and symmetry of the distributions of bone and cartilage despite marked differences in the relative position of the fragments from specimen to specimen, and the intimacy with which bone and cartilage were mixed in certain situations, made it difficult to ascribe cartilage formation to the operation of mechanical determinants, at least in the early part of repair, while the vascular hypothesis satisfactorily explained all the appearances.

On the other hand, the situation of the later-appearing parosteal cartilage in the femoral fractures in the rat did appear to be closely determined by the position of the fragments, and the mechanical hypothesis in this instance received support.

We do not attempt, therefore, to refute the view that fibrous connective tissues can be induced to undergo chondrification in the presence of certain types of mechanical stress, for which indeed there is direct experimental evidence (Glücksmann, 1939); or that this transformation may explain the production of *some* of the cartilage in certain types of fracture, e.g. where immobilization is poor, but our evidence does suggest that the vascular hypothesis, rather than the mechanical one, accounts most satisfactorily for the cartilage which appears at an early stage in the periosteal blastema.

The poverty of periosteal cartilage production in the repair of the skull, and in incomplete saw-cut fractures of the long bones is, in our view, explicable in terms of the reduced volume of blastemal cells which appear in such circumstances, and the consequent ability of new blood-vessel formation to keep pace with the cellular proliferation.

Finally, the vascular and mechanical hypotheses are not necessarily antagonistic,

even as an explanation for the transformation of parosteal fibrous tissue into cartilage in the later stages of repair, for it is quite likely that shearing and pressure stresses acting on connective tissue would tend to inhibit the ingrowth of blood vessels, or destroy those already present, and so predispose towards cartilage formation.

Whatever its origin, however, the adaptational value of cartilage during fracture repair cannot be doubted, for owing to its relative independence of blood supply, it can speedily restore some rigidity to the skeleton before the new bone, dependent on a rich blood supply, has bridged the gap. In this connexion it is significant that in the frog and lizard and in the tibial fractures in the rat, where periosteal cells were chiefly responsible for the organization of the haematoma, primary cartilaginous union always occurred, whereas in the femoral fractures in the rat, where parosteal cells were chiefly involved, fibrous union was the rule. This clear-cut distinction at once indicates the specificity and importance of the periosteal cells, and stresses the inadequacy of the mechanical hypothesis solely to account for the formation of cartilage in fracture repair, unless one makes the *ad hoc* hypothesis that the periosteal cells are much more sensitive to mechanical stresses than ordinary fibroblasts.

Calcification of cartilage has also been ascribed to mechanical influences, and has been given an adaptational significance (Erdheim's (1914) 'calcio-protective law'). Wallis (1928), in his experiments on lizards, describes a long delay in the replacement of fracture cartilage by new bone, the cartilage meanwhile becoming heavily calcified, and following Erdheim he attributes this to the need for strengthening the bond of union between the fragments. We did not find such a delay, however, possibly because of the higher temperature employed, and moreover we found that calcification of cartilage was an accompaniment of endochondral ossification, and did not occur except in the vicinity of the eroding blood vessels, the bulk of the cartilage before ossification began being uncalcified. The reason for this discrepancy in Wallis's and our results is not clear.

Harris (1933) takes a diametrically opposite view to ours as to the relationship of calcification in cartilage to vascularity, for he regards calcification as the result of ischaemia. It must be pointed out, however, that calcified cartilage is found in the growing skeleton in three main situations, viz. at the centre of ossification in the shaft, in the deepest zone of the articular cartilage, and in the hypertrophic cartilage of the epiphyseal plate, in all of which situations it is nearer blood vessels, viz. those of the vascular periosteal bud, of the bony epiphysis and of the metaphysis, than is the uncalcified cartilage.

Specific differences

Generally speaking, the lizard occupied an intermediate position between the rat and the frog in respect of most of the differences found, though, with two exceptions, the lizard was more like the rat than the frog.

The differences were both quantitative and qualitative. The chief quantitative differences were: (1) in the rates of repair, which may be ascribed to such factors as temperature, and specific differences in metabolic rates; (2) in the relative amounts of periosteal and parosteal blastemal tissues produced in the course of repair, which may be ascribed to specific differences in the ease with which these tissues respond to the fracture stimulus; and (3) in the relative amounts of cartilage and bone formed

by the periosteal blastema, which appeared to be correlated with the richness or otherwise of the blood supply although the reasons for the differences in vascularity remain obscure. In this last respect the lizard and the frog agreed in having a poorly vascularized periosteal blastema, whereas in the rat the vascularity was very much greater.

In the frog the extreme slowness of endochondral bone formation, the difficulty with which alkaline phosphatase activity could be demonstrated, and the virtual absence of calcification in the cartilage, separated this animal sharply from the other two and constituted quantitative differences so gross that they almost partook of a qualitative character, making the differences in these respects between the rat and lizard seem trivial by comparison.

The qualitative differences chiefly concerned the methods of cartilage erosion and endochondral replacement, and the behaviour of the cartilage in the neighbourhood of the erosion cavities.

As far as the actual erosion was concerned, the rat and lizard differed chiefly in respect of the regularity and size of the excavations, differences which were paralleled at the normal epiphyseal lines of the two animals. They agreed in that erosion began peripherally and was accompanied by the appearance of numerous multinucleated chondroclasts. In the frog, however, erosion chiefly occurred around existing cartilage canals, and was unaccompanied by chondroclastic activity. In the rat and lizard the cartilage was of the hypertrophic variety and was calcified prior to its resorption; in the frog, on the other hand, the cartilage was of the normal hyaline type and did not become hypertrophic or calcified prior to its removal.

It may here be stated that in all probability these differences in the behaviour of the cartilage are interdependent. It is generally believed that hypertrophic cartilage is more easily calcified than hyaline, and that chondroclasts do not appear during the resorption of cartilage unless it is calcified (Weinmann & Sicher, 1947). The virtual absence of phosphatase from frog's cartilage is perhaps also related to its hyaline character, for it is well known that in mammalian cartilage this enzyme is only found in the hypertrophic variety. It is significant in this connexion that in the long bones of the frog, endochondral ossification does not occur (Haines, 1942) and the epiphyseal cartilage during growth in length is resorbed without hypertrophic changes, calcification, or the appearance of chondroclasts.

The most important difference between the species, however, concerned the behaviour of the cartilage in the neighbourhood of the erosion cavities. Here the frog and the lizard showed several common features which were not found in the rat. These features have already been fully described in the presentation of the results, where the conclusion was drawn that an actual transformation of cartilage into bone was taking place.

The possibility of such a transformation has alternatively been affirmed and denied in the past. Thus on evidence essentially similar to ours, Wurmbach (1927), denies that, in the newt, an actual transformation is taking place and interprets the appearances as accompaniments of calcification, although in the frog, in our experiments, calcification did not occur, and in any case the appearances were too striking to be dismissed so lightly. Ziegler (1899) and Fujinami (1901), however, express

themselves strongly in favour of an actual conversion of cartilage into bone in amphibian fracture repair.

Weidenreich (1930), in mammals, admits that cartilage cells whose capsules have been partially eroded during the course of normal endochondral ossification may become transformed into osteoblasts and begin to deposit new bone matrix within the remaining parts of the capsules, but he does not regard this as evidence of metaplasia. The appearances we described were not of this character, however, but were more like those described by Mjassojedoff (1922) in the course of ossification of the hen's tracheal rings, which he interpreted as a direct transformation of cartilage into bone; and by Ziba (1910), in the foetal petrous temporal bone of man, who interpreted the appearances similarly. Koelliker (1889) states that cartilage is converted directly into bone in the recovery stage of human rickets and his illustrations are strongly reminiscent of our observations on the lizard. Perhaps the best evidence, however, comes from the tissue culture experiments of Fell (1933) and Roulet (1935), who found that the cartilage developing in explants of embryonic bony tissue is unstable, and becomes converted directly into typical bony tissue.

A priori, such a transformation should not be surprising, in view of the evidence given above for the common origin of osteoblasts and chondroblasts in the periosteal blastema, and when it is remembered that the organic matrices of bone and cartilage have a common basis of collagen fibres, and differ only in the type of cementing substance deposited around the fibres, the so-called 'ossein' of bone and 'chondromucin' of cartilage. Leaching out of the chondromucin, such as seems normally to occur at articular surfaces, and at the medullary extremity of the epiphyseal cartilage in the frog, should therefore leave behind a collagenous framework eminently suitable for subsequent impregnation with ossein to give bone matrix, especially if, at the same time, the irruption of blood vessels into the vicinity of the cartilage cells should stimulate their conversion into bone cells.

Finally, it should be pointed out that this apparent conversion of cartilage into bone is not a phenomenon peculiar to fractures in the frog and lizard, but occurs, although not on so extensive a scale, at the normal epiphyseal lines of the lizard's femur, and in the frog's acetabulum.

As a general rule, therefore, we may state that where qualitative differences exist between the species as regards the course of fracture repair, they reflect specific behaviour characteristics of the normal skeletal tissues, and with this in mind we may perhaps characterize fracture repair from a general biological standpoint, as an expression of inherent patterns of growth and differentiation superimposed on a non-specific reaction of the bony tissues to trauma.

SUMMARY

1. The repair of experimental closed fractures has been investigated histologically in the femora of the frog, lizard and rat, and in the tibia of the rat.

2. In each series a reparative blastema was formed in the early stages by the proliferation of periosteal, medullary and intermuscular (parosteal) connective tissue cells around the haematoma at the fracture site. The haematoma was later organized by cells growing in from these three sources.

3. New bone, cartilage and an intermediate type of tissue were early deposited in

the periosteal blastema; later, new bone appeared in the medullary blastema; while the parosteal blastema developed at first like an ordinary granulation tissue. The periosteal blastema was the most widespread, the medullary the least so.

4. In the rat more bone than cartilage developed in the periosteal blastema, but in the frog and lizard cartilage was the dominant tissue.

5. In the lizard and rat the periosteal and medullary blastemata showed intense alkaline phosphatase activity which preceded calcification of the bone matrix by a relatively short interval, but calcification of the cartilage by a considerable interval of time. Phosphatase activity was difficult to demonstrate in the frog.

6. In the frog and lizard, the haematoma was organized chiefly from the periosteum, and cartilaginous union readily occurred in spite of gross angulation and overlapping of the fragments in some cases. In the rat the parosteal blastema played a more important role, particularly in the femoral fractures, where displacement of the fragments was considerable, and fibrous union preceded a long-delayed secondary cartilaginous union. In the tibial fractures displacement was relatively slight, and primary cartilaginous union was the usual outcome.

7. Replacement of cartilage by bone took place in a different manner in each species. In the rat chondroclasts, blood vessels and osteoblasts rapidly advanced into the cartilage from each side in a regular manner as in endochondral ossification at the epiphysial line. In the lizard the invasion occurred less rapidly, and in an irregular manner from all sides. The resulting vascular spaces were wider, and the endochondral bone trabeculae coarser, than in the rat. In the frog the cartilage was eroded very slowly, chiefly from within, by the enlargement of pre-existing vascular canals, on whose walls bone matrix was later deposited. Chondroclasts in the frog were very rarely seen.

8. In the frog and lizard, but not in the rat, the uneroded cartilage appeared to become transformed directly into bone, for its matrix lost its basophilia and became increasingly fibrous and eosinophil, while its cells shrank to the shape and dimensions of osteocytes.

9. In all species there was some lag in the calcification of the bone matrix, for the newly formed trabeculae had uncalcified borders. In the rat and lizard calcification of the cartilage accompanied the ingrowth of blood vessels, and only occurred in their neighbourhood. In the frog the cartilage was virtually uncalcified except to a variable and slight degree in the immediate neighbourhood of the vascular canals. This is possibly attributable to the low phosphatase activity of the cartilage in this animal.

10. From the results it was concluded (*a*) that osteoblasts and chondroblasts are differentiated from similar cells in the periosteal blastema, the former appearing where the blood supply is adequate, the latter where it is deficient, (*b*) that the parosteal connective tissue cells may differentiate into chondroblasts when acted upon by certain types of mechanical stress, but ordinarily they remain as fibroblasts.

11. On the basis of the observed similarities and differences between the species it was concluded that the skeletal tissues during fracture repair exhibit specific modes of growth and differentiation superimposed on a generalized and non-specific reaction to trauma.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Lizard. 5 days. Unresolved haematoma. Early periosteal and parosteal reactions. Weigert's haematoxylin and van Gieson. ×21.

Fig. 2. Lizard. 11 days. Early cartilage formation in periosteal blastema (cf. fig. 12). Weigert's haematoxylin and van Gieson. ×21.

Fig. 3. Lizard. 14 days. Incipient cartilage union, repair of periosteum and formation of an external bony shell (cf. fig. 11). Weigert's haematoxylin and van Gieson. ×21.

Fig. 4. Lizard. 21 days. Cartilage union and early stage of vascular erosion. Weigert's haematoxylin and van Gieson. ×21.

Fig. 5. Lizard. 31 days. Established bony union. Weigert's haematoxylin and van Gieson. ×21.

Fig. 6. Lizard. 7 days. Alkaline phosphatase in periosteal blastema. Gomori. ×112.
Fig. 7. Lizard. 21 days. Vascular erosion of cartilage. Weigert's haematoxylin and van Gieson. ×112.
Fig. 8. Lizard. 7 days. Alkaline phosphatase in young cartilage of periosteal blastema. Gomori. ×490.
Fig. 9. Lizard. 16 days. Calcification in cartilage. Cobalt sulphide. ×490.
Fig. 10. Lizard. 5 days. Collagenous and precollagenous fibres of the periosteal blastema. Wilder. ×490.
Fig. 11. Lizard. 14 days. Incipient cartilage union (cf. fig. 3). Methylene blue and eosin. ×21.
Fig. 12. Lizard. 11 days. Early cartilage formation in periosteal blastema (cf. fig. 2). Weigert's haematoxylin and van Gieson. ×112.
Fig. 13. Lizard. 7 days. Bone formation in the medullary cavity. Haematoxylin and eosin. ×112.

PLATE 2

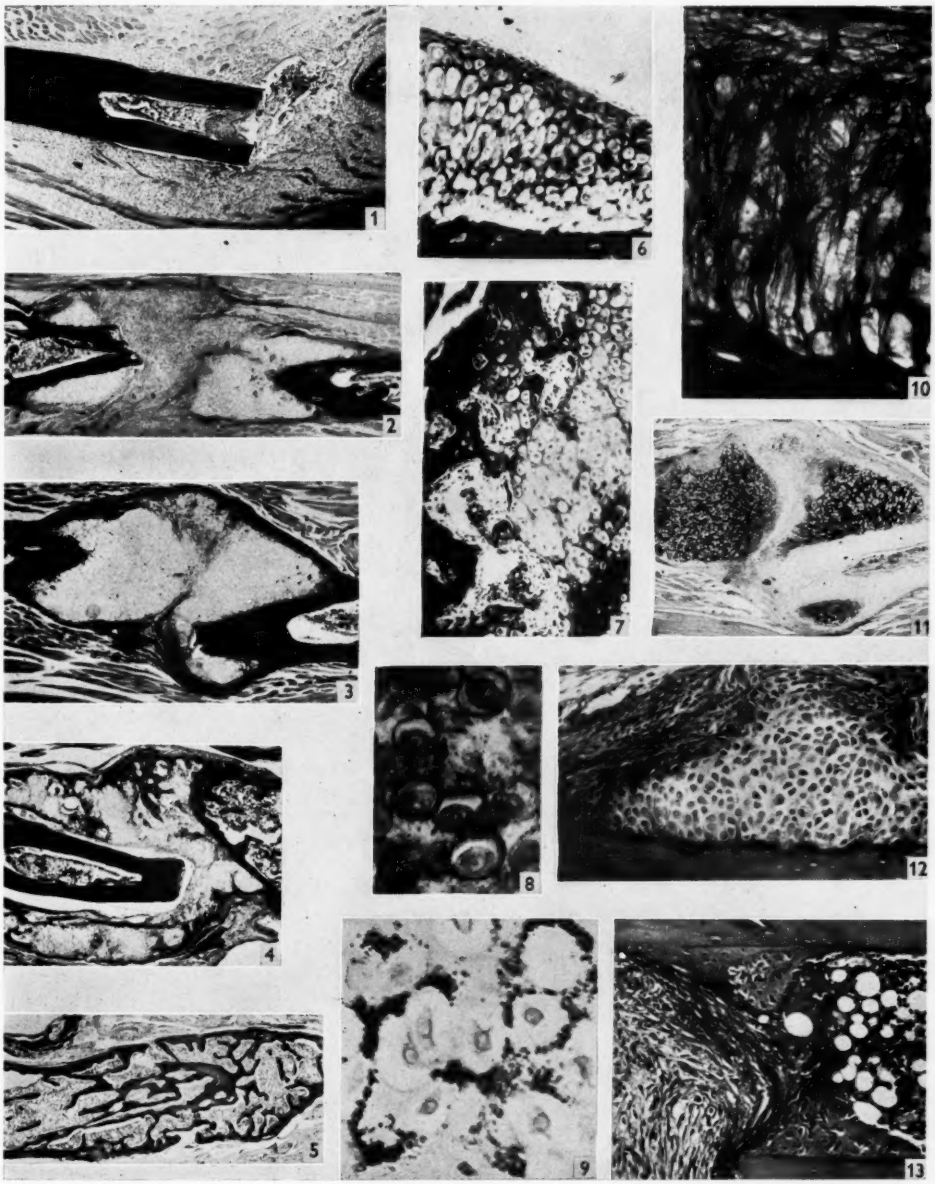
Fig. 14. Lizard. 21 days. Loss of basophilia and appearance of eosinophilia in cartilage near eroding blood vessels. Methylene blue and eosin. ×112.
Fig. 15. Lizard. 21 days. Apparent transformation of cartilage into bone. Weigert's haematoxylin and van Gieson. ×490.
Fig. 16. Frog. 11 days. Early periosteal reaction. Weigert's haematoxylin and van Gieson. ×17·5.
Fig. 17. Lizard. 25 days. Endochondral bone trabeculae. Calcification of central cores of residual cartilage. Uncalcified lamellar bone at surface of trabeculae. Cobalt sulphide. Weigert's haematoxylin and van Gieson. ×112.
Fig. 18. Frog. 11 days. Early periosteal blastema. Weigert's haematoxylin and van Gieson. ×112.
Fig. 19. Frog. 21 days. Vascular canals in cartilage. Methylene blue. ×112.
Fig. 20. Frog. 8 days. Early periosteal blastema. Note osteoblasts on surface of old bone. Pyronin and methyl green. ×490.
Fig. 21. Frog. 11 days. Early periosteal blastema. Weigert's haematoxylin and van Gieson. ×490.
Fig. 22. Frog. 70 days. Cartilage union. Weigert's haematoxylin, van Gieson and methylene blue. ×21.

PLATE 3

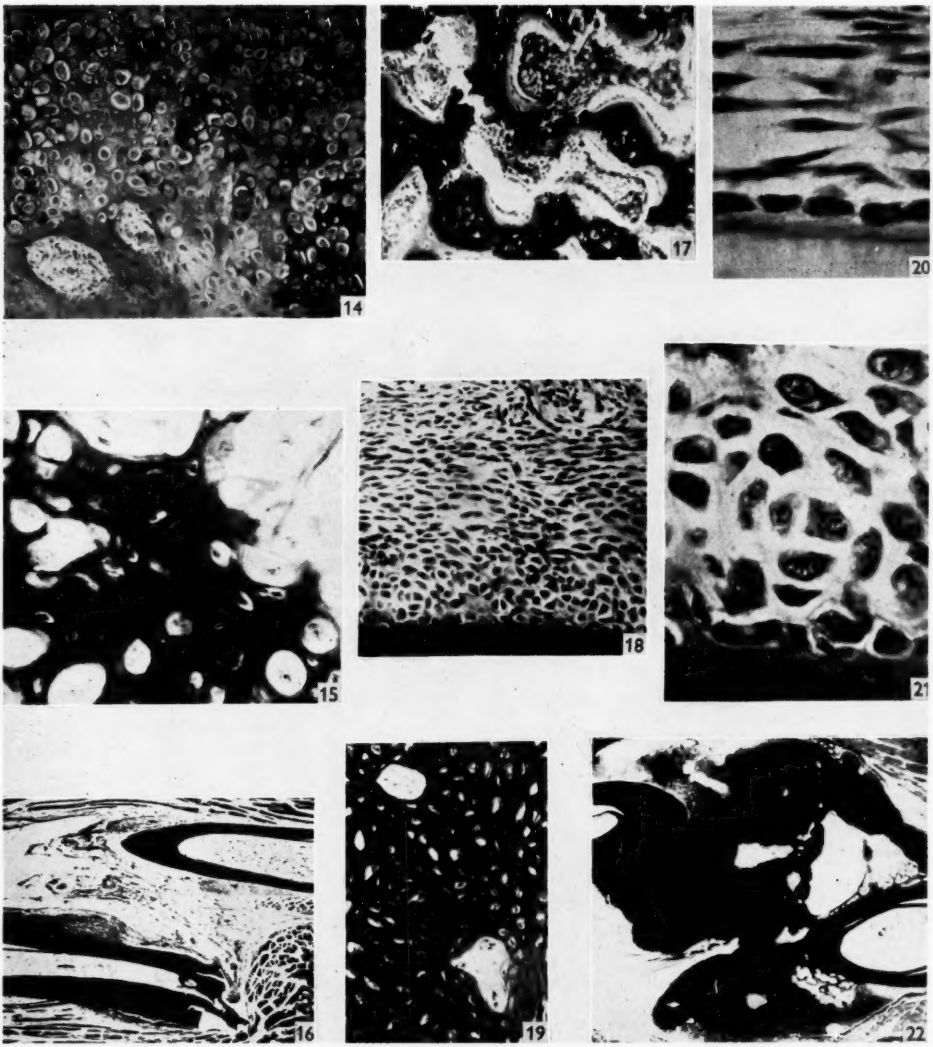
Fig. 23. Frog. 30 days. Bone formation in cartilage canals. Fibrosis of cartilage matrix. Weigert's haematoxylin, van Gieson and methylene blue. ×112.
Fig. 24. Frog. 30 days. Loss of basophilia of cartilage around vascular canals. Early bone formation in walls of canals. Weigert's haematoxylin, van Gieson and methylene blue. ×112.
Fig. 25. Frog. 36 days. Shows changes in and around a cartilage canal. From without inwards note (1) normal basophil cartilage, (2) non-basophil cartilage, (3) highly calcified new bone matrix, (4) uncalcified new bone matrix, (5) osteoblasts, (6) primitive connective tissue. Cobalt sulphide and methylene blue. ×490.
Fig. 26. Frog. 30 days. Shows loss of basophilia preceding fibrosis of cartilage and its apparent transformation into bone. Weigert's haematoxylin, van Gieson and methylene blue. ×112.
Fig. 27. Frog. 70 days. Shows apparent transformation of cartilage into bone between vascular canals, and direct bone deposition in walls of canals. Weigert's haematoxylin, van Gieson and methylene blue. ×112.
Fig. 28. Rat femur. 7 days. Note periosteal new bone and pre-cartilage; medullary new bone; parosteal granulation tissue and the haematoma cavity. Weigert's haematoxylin and van Gieson. ×17·5.

PLATE 4

Fig. 29. Rat femur. 5 days. New bone and pre-cartilage in greatly thickened periosteum. Weigert's haematoxylin and Van Gieson. ×70.
Fig. 30. Rat femur. 24 days. Chondrification and endochondral ossification advancing into dense connective tissue between fragments (cf. fig. 33). Weigert's haematoxylin and van Gieson. ×21.
Fig. 31. Rat tibia. 4 days. Alkaline phosphatase in periosteal blastema. Gomori. ×112.
Fig. 32. Rat femur. 5 days. Shows transitions from bone, through an intermediate tissue to cartilage in the periosteal blastema. Weigert's haematoxylin and van Gieson. ×112.
Fig. 33. Rat femur. 24 days. High power view of part of section shown in fig. 30. Shows endochondra bone, cartilage and fibrous tissue. Methylene blue and eosin. ×112.
Fig. 34. Rat tibia. 4 days. Alkaline phosphatase in fracture gap at junction of periosteal (left) and parosteal (right) blastemata. Gomori. ×112.



PRITCHARD AND RUZICKA—COMPARISON OF FRACTURE REPAIR IN THE FROG, LIZARD AND RAT



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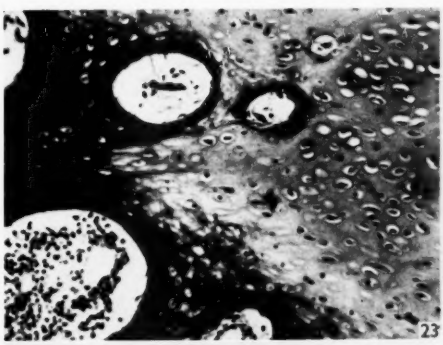

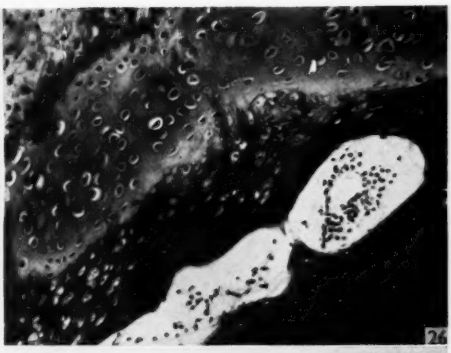

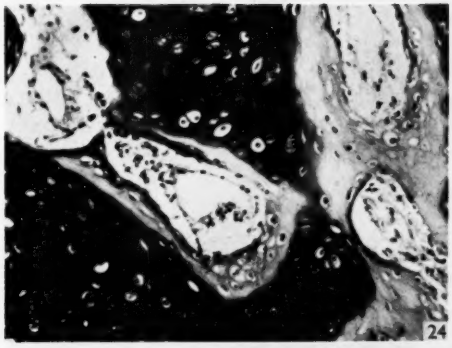

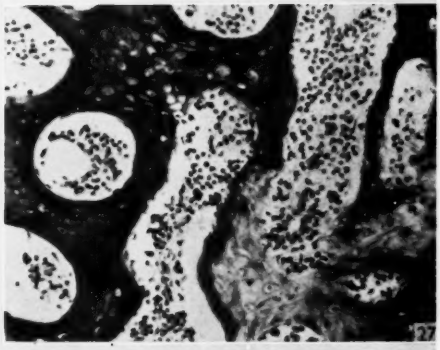

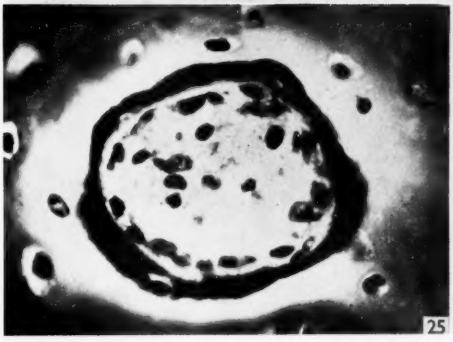

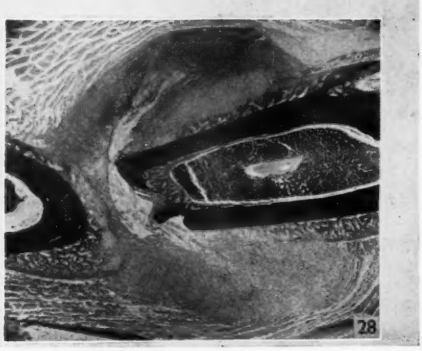

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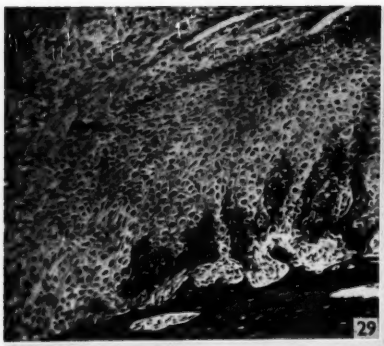

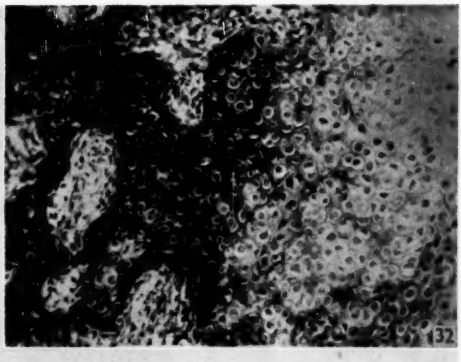

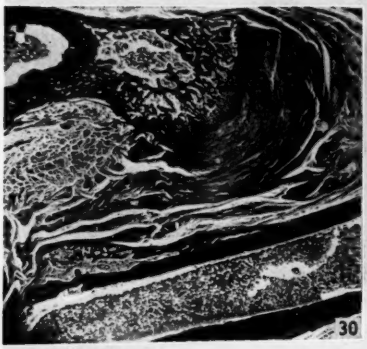

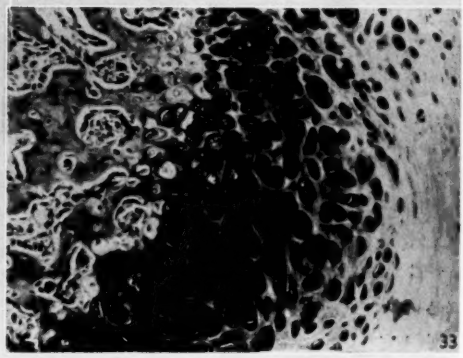

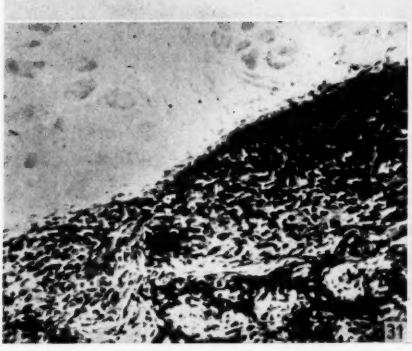

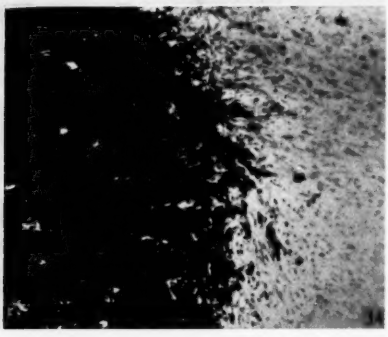

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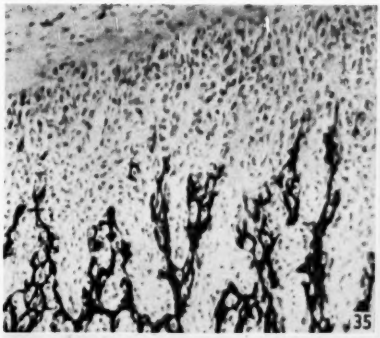
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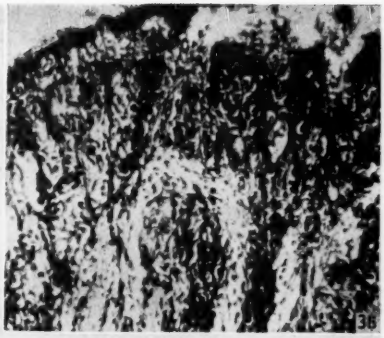
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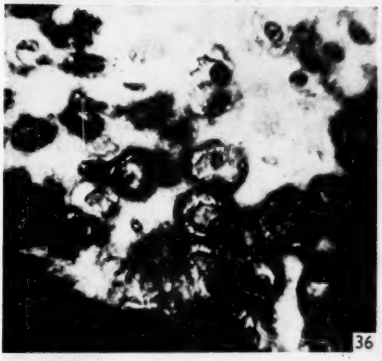
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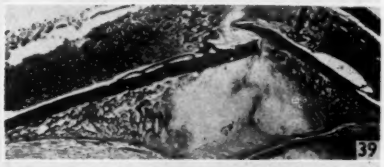
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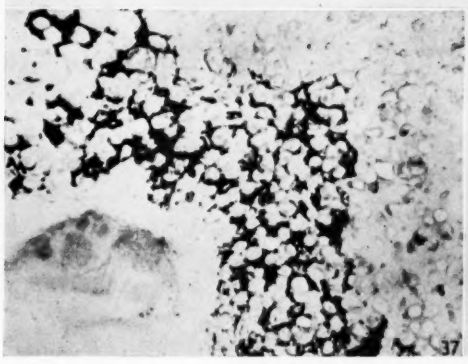
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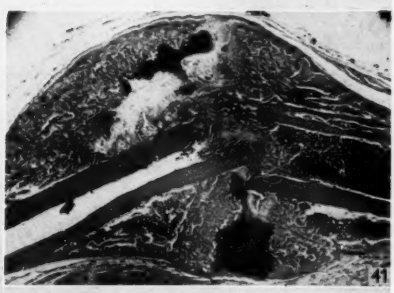
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PLATE 5

Fig. 35. Rat tibia. 4 days. Calcified new bone trabeculae in deeper layers of periosteal blastema. Cobalt sulphide (cf. fig. 38). ×112.

Fig. 36. Rat tibia. 4 days. Alkaline phosphatase in newly formed cartilage of periosteal blastema. Gomori. ×490.

Fig. 37. Rat tibia. 12 days. Calcified cartilage around a blood vessel. Cobalt sulphide and eosin. ×112.

Fig. 38. Rat tibia. 4 days. Alkaline phosphatase in periosteal blastema. To be compared with fig. 35 which shows an adjacent section stained for calcium salts. Gomori. ×112.

Fig. 39. Rat tibia. 10 days. Cartilage union. Weigert's haematoxylin and van Gieson. ×17·5.

Fig. 40. Rat tibia. 12 days. Indian ink injected specimen. Cartilage union with endochondral bone formation on either side. Haematoxylin and eosin. ×21.

Fig. 41. Rat tibia. 17 days. Relatively advanced stage in repair. Shows bony and cartilaginous union. Weigert's haematoxylin, van Gieson and methylene blue. ×17·5.

EFFECT OF MALNUTRITION AS COMPARED WITH HYPOPHYSECTOMY ON ORGAN WEIGHT OF THE ALBINO RAT

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INTRODUCTION

Changes in organ weight after prolonged malnutrition resemble in some ways those found after hypophysectomy. This, and the fact that loss in organ weight after inanition can be partially overcome by injecting pituitary extracts, has suggested the possibility that the action of malnutrition on organ weight may be exerted indirectly through inhibition of pituitary secretion. The term 'pseudohypophysectomy' has been coined to describe this condition.

On the other hand, hypophysectomized animals eat less than normal controls and thus, some of the changes brought about by hypophysectomy could be attributed to underfeeding.

The scope of this paper is the comparison of organ weights of young albino rats subjected to various restricted diets for 30 days with those found in hypophysectomized rats of the same initial weight 30 days after operation.

MATERIAL AND TECHNIQUES

Forty-eight male and forty-eight female 100 g. ($\pm$ 10) albino rats were used in these experiments. Eight animals of each sex were killed at the beginning of the experiment and were used as initial controls. The rest were kept for 30 days in individual cages and each group of sixteen rats (eight males, eight females) received a low calorie diet as shown in Table 1*a*. Group V—receiving bread and milk *ad lib.*—were used as 'final controls'.

Table 1*a*

Group	I	II	III	IV	V
Bread (g.)	2	3	4	10	*ad lib.*
Milk (g.)	4	6	8	20	*ad lib.*
Cal.	9	13·5	18	44·5	*ad lib.*

Calorie value was calculated on the usual type of bread and milk used in Buenos Aires. Water intake was not restricted.

The vitamin content of the diets (Table 1*b*) has been calculated from the values for bread and milk given by the National Institute of Nutrition, Buenos Aires.

Table 1*b*

Vitamin	A	B_1	B_2	Niacin	C	D
	(i.u.)	(γ)	(γ)	(γ)	(γ)	(i.u.)
Diet 1	11·8	5·4	12·12	53·28	40	0·204
Diet 2	17·5	8·1	18·18	79·92	60	0·306
Diet 3	23·6	10·8	24·24	106·56	80	0·408
Diet 4	59·0	27·0	60·6	266·4	200	1·020

The animals were weighed every other day, and at the end of the experiment were killed with coal gas. Then the total length was measured, from nose to tip of tail and the weight of heart, spleen, liver, kidneys, thymus, thyroid, adrenals, testes, prostate, seminal vesicles, coagulating glands, ovaries and uterus was measured, the bilateral organs being weighed together.

RESULTS

Mortality and morbidity. Only six rats died; one of each sex in group I and two of each in group II. On the whole, the animals seemed to be fairly resistant to restricted calorie intake, not showing signs of depression or exhaustion, even with a weight loss of about 30 %, but on the contrary an increase in activity in an instinctive search for food. Only in some cases was coma observed at the end of the experiment. No oedema could be detected on physical examination.

Total body weight

With diets 1 and 2 (Figs. 1, 2) both males and females lost weight gradually and progressively from the first to the last day of the experiment. The curve for females is more or less the same with both diets, but the male curve has a slightly more

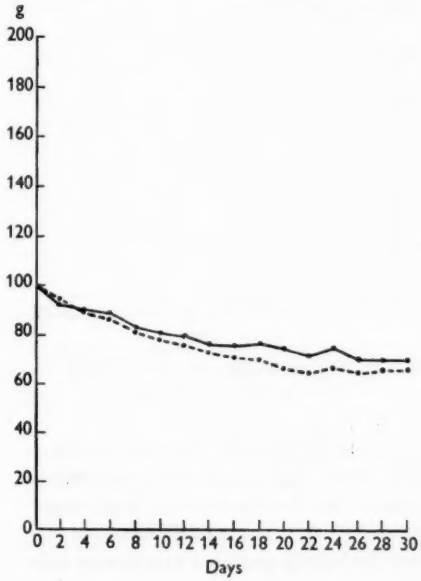

Fig. 1. Total weight curve under diet 1.
——, males; - - - -, females.

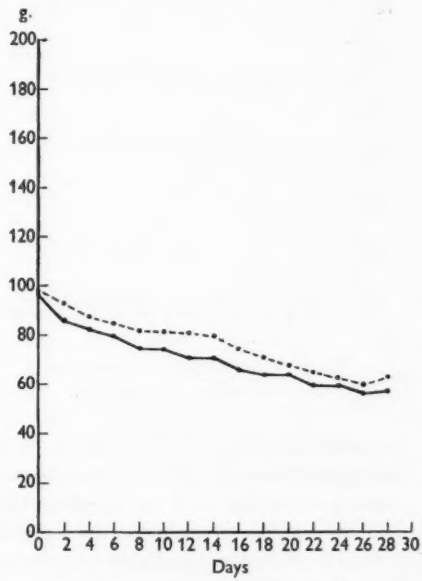

Fig. 2. Total weight curve under diet 2.
——, males; - - - -, females.

pronounced slope in group II, in spite of the higher calorie intake as compared with group I (see discussion). With diet 3 (Fig. 3) this curve is nearly horizontal, showing that this diet (18 cal./day) is just sufficient to prevent growth without actual loss in body weight, that is, intake and output are in equilibrium. However, the females

show a gradual loss, then a plateau, and finally recover their initial weight, probably through metabolic adaptation to low calorie intake.

Diet 4—44·5 cal./day—seems to provide the necessary calorie requirements for normal growth, since the growth curves (Fig. 4) and organ weights for this group are similar to those of the 'final controls' (group V, Fig. 5) receiving food *ad lib.* The rate of normal growth is higher for the males, a fact that has been repeatedly observed in the albino rat. Fig. 6 shows the variations in body weight in percentage of initial weight for the different diets employed.

Total length (Tables 2 and 3). The length remains more or less constant in groups I and II and increases slightly in group III. Specially noteworthy is the difference

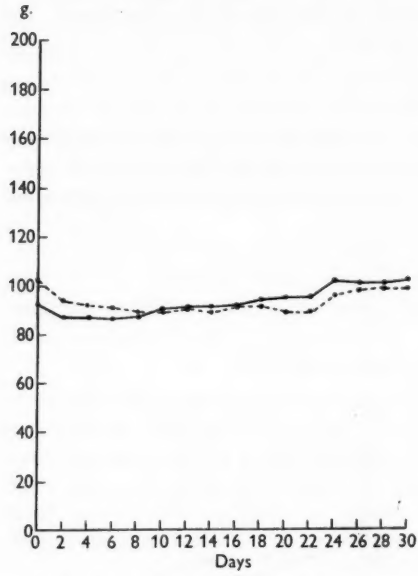

Fig. 3. Total weight curve under diet 3.
——, males; - - - -, females.

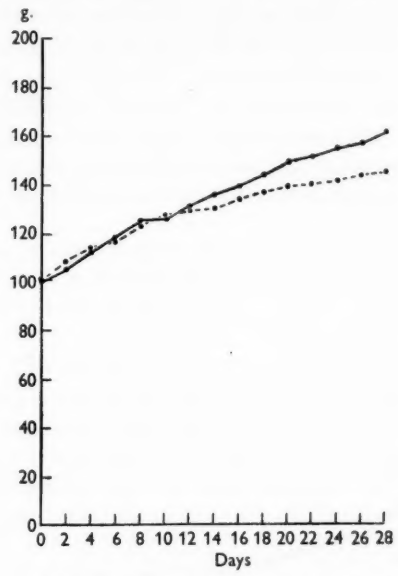

Fig. 4. Total weight curve under diet 4.
——, males; - - - -, females.

between the changes in total length—which represents roughly the skeletal growth—and those in body weight, i.e. total growth. In the two first groups the body weight decreases markedly while the head/vertebral column/tail length remains unchanged and even increases with diet 3 in spite of the total growth being at a standstill.

On the other hand, with the unrestricted diets the length increases at a slower rate than the weight.

Thymus (Tables 2 and 3). The thymus weight is more strikingly affected by malnutrition than that of any other organ. With low calorie diets, 1 and 2, it decreases in 30 days down to 5 % of its initial weight. Decrease in weight is still evident with diet 3.

The fact that thymus weight is higher in group IV than in group V, is indicative of the individual variations in thymus weight exhibited by normal animals.

Spleen (Tables 2 and 3). The changes in the weight of this other lymphoid organ are parallel with those of the thymus though less marked, losing approximately 40 % of its initial weight with diet 1 in both sexes. Diet 2 produces an even greater loss in the males than diet 1, but only a slight loss in the females. Similarly, in group III there is a 10 % decrease in spleen weight for the males and a corresponding 10 % increase among the females. These figures seem to suggest that the spleen has a greater sensitivity to inanition in the male sex.

Liver, heart and kidneys (Tables 2 and 3). These three organs are modified in a similar way. In the male rats they undergo significant atrophy with diets 1–3 and in the females with diets 1 and 2. Diet 3 prevents growth of liver and kidneys in the females but allows the heart to increase its initial weight by more than 20 %. Both diets 4 and 5 permit increase in weight of liver, heart and kidney in both sexes.

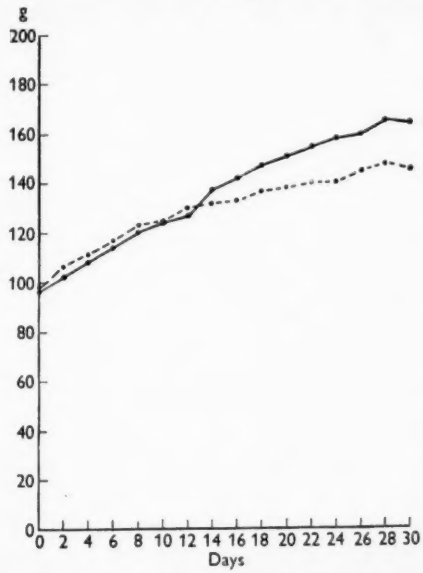

Fig. 5. Total weight curve under diet 5. ——, males; - - - -, females.

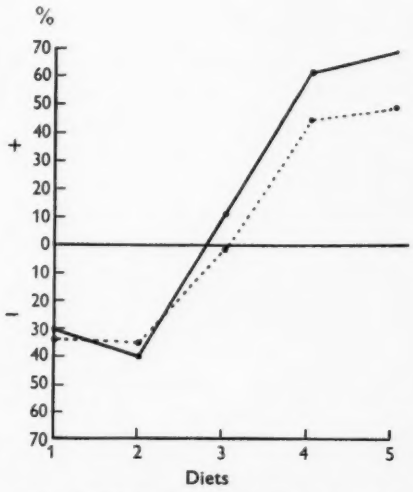

Fig. 6. Weight variations in percentage of initial weight under different diets.

Thyroid (Tables 2 and 3). There is slight atrophy with diets 1 and 2, slight growth with diet 3, and marked growth with diets 4 and 5.

Adrenals (Tables 2 and 3). The adrenal glands show little or no atrophy even with the more restricted diets 1 and 2. Initial weight is maintained with diet 3 and growth proceeds normally under diets 4 and 5. The loss of weight in groups I and II is slightly greater among the females than among the males.

Testes and accessory organs (Table 2). Growth of the testes persists with all the diets employed. The prostate maintains its weight under diet 1, grows slowly under diet 2 and rapidly with diets 3–5. The seminal vesicles and the coagulating glands grow with all the diets except in group II (see discussion). The great growth of the sex organs observed with diets 4 and 5 is due to puberty.

Table 2. *Length in millimetres, and weight in milligrams of organs of male albino rats after 30 days of underfeeding with diets 1–5. Each figure represents the mean of eight observations*

Males	Length	Thymus	Spleen	Liver	Heart	Kidneys	Thyroid	Adrenals	Testes	Prostate	Sem. ves.	Coag. gl.
Initial controls	*280*	*234*	*540*	*5360*	*542*	*992*	*8·0*	*21·0*	*804*	*40*	*22*	*5·5*
Group I (2 g. bread, 4 g. milk)	287	33	347	2974	379	807	7·5	19·7	1350	40	24	6·5
Group II (3 g. bread, 6 g. milk)	279	13	173	2250	294	747	6·0	20·0	915	43	16	3·5
Group III (4 g. bread, 8 g. milk)	295	134	473	4533	415	895	9·5	22·7	1968	106	147	20·0
Group IV (10 g. milk) 20 g. milk)	340	294	—	8410	736	1360	22·8	34·6	2040	111	191	34·0
Group V (*ad lib.* controls)	336	229	1060	8972	914	1779	21·0	33·7	2174	217	338	51·0

Table 3. *Length in millimetres, and weight in milligrams of organs of female albino rats after 30 days of underfeeding with diets 1–5. Each figure represents the mean of eight observations*

Females	Length	Thymus	Spleen	Liver	Heart	Kidneys	Thyroid	Adrenals	Ovaries	Uterus
Initial controls	*279*	*208*	*498*	*4522*	*418*	*944*	*9*	*25*	*29*	*161*
Group I (2 g. bread, 4 g. milk)	277	56	286	2836	296	720	7	20	19	43
Group II (3 g. bread, 6 g. milk)	281	15	460	2700	318	750	7	22	23	47
Group III (4 g. bread, 8 g. milk)	295	196	546	4680	517	957	10	26	29	82
Group IV (10 g. bread, 20 g. milk)	325	250	844	7400	800	1455	21	43	70	284
Group V (*ad lib.* controls)	319	286	1027	6654	700	1315	21	43	63	314

Ovaries and uterus (Table 3). The ovary seems to be more sensitive to malnutrition than the testis, since it loses weight with diets 1 and 2 and only succeeds in keeping a constant weight with diet 3. The rate of growth with diets 4 and 5 is very high as one would expect in normal puberty. The uterus undergoes atrophy with diets 1–3 in a proportion far greater than the ovary. Growth under normal calorie intake is correspondingly smaller than that of the ovary.

EFFECT OF HYPOPHYSECTOMY

The effects of 30 days malnutrition on organ and body weight were compared with the figures obtained 30 days after hypophysectomy by H. Houssay* in 100 g. albino rats of the same strain.

* 'Hipofisis y Crecimiento' (doctorate thesis). Universidad Nacional de Buenos Aires, 1947.

The parapharyngeal technique of P. Smith modified by Selye was used for the operations, the complete ablation of the pituitary gland being verified at autopsy.

Comparison with hypophysectomized rats submitted to forced feeding was made in order to judge to what extent the degree of organ atrophy after operation was due to decreased food intake.

The body weight curve of hypophysectomized animals (Fig. 7) lies between that obtained with diets 2 and 3. After forced feeding average body weight of hypophysectomized animals reaches 110 g. in males and 119 g. in females.

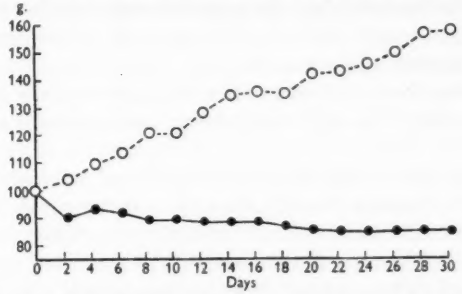

Fig. 7. Total weight curve of hypophysectomized rats. ●—●, operated animals; ○--○, controls.

Table 4. *Organ weight in percentage of initial weight after* 30 *days*

(The figures under 'Inanition' represent the minimum weights obtained (either with diets 1 or 2).

Males	Hypophysectomy	Inanition	Hypophysectomy forced feeding
Length	107	98	
Thymus	80	5	76
Liver	88	42	112
Spleen	48	32	92
Heart	66	54	113
Kidneys	44	75	85
Thyroid	41	75	92
Adrenals	39	94	50
Testes	13	114	35
Prostate	11	98	22
Sem. ves.	13	69	12
Coag. gl.	18	64	24

Table 5. *Organ weight in percentage of initial weight after* 30 *days*

Females	Hypophysectomy	Inanition	Hypophysectomy forced feeding
Length	100	100	
Thymus	73	5	75
Liver	70	60	181
Spleen	49	57	84
Heart	75	71	91
Kidneys	57	76	78
Thyroid	63	77	56
Adrenals	40	82	43
Ovaries	28	67	32
Uterus	18	27	74

The effect of hypophysectomy is especially marked on the gonads, adrenals, thyroid and kidneys, the atrophy of these organs one month after operation being

greater than the greatest atrophy obtained by inanition, even with those diets (1 and 2) which produce a greater loss in total body weight than is caused by pituitary ablation (Tables 4 and 5).

On the other hand, some organs are less affected by lack of pituitary secretion than by lack of food, notably thymus and liver.

DISCUSSION

In examining the data it must be borne in mind that these experiments were done in young rapidly growing animals in the pubertal stage, so that mere retardation of growth, even if organ weight does not fall below the initial figures, is significant, particularly so in the case of the gonads.

Body weight curves show that under restricted calorie intake females decrease in weight more than males, thus exhibiting like the normal rats, a sexual difference in growth impulse.

Thermal and seasonal variations have a significant influence upon the rate of growth. According to Campbell (1945), for rats receiving equal amounts of food the rate of growth is higher during the warmer months due to better utilization. This may be the explanation for the larger figures obtained with diet 1 as compared with diet 2 for body weight and for the weight of various organs as well, since group I was placed on restricted diet in a warmer period than group II. ('Effective temperature' for both periods was calculated from official 3 hr. meteorological data.)

We have seen that variation in *length* is quite small compared with variation in body weight, suggesting that skeletal growth is largely independent of quantitative variations in diet. This view is supported by the results of McLennan & Jackson (1933) who found a decrease of only 5–7 % in the weight of humerus and femur in underfed rats that had lost up to 40 % of their body weight, and those of Stewart (1918) who found normal skeletal growth in newly born rats whose body weight was maintained at a standstill by intermittent interruption of lactation.

Jackson, Stewart and others have reported that tail length decreases less than the vertebral column length, underfed rats being comparatively 'long-tailed' animals. However, the exact proportions are difficult to establish accurately, due to a certain degree of kyphosis present in many of the underfed rats, which has also been observed by Denton & Kohman (1918).

The marked and constant loss of weight undergone by the *thymus* might be attributed to summation of effects of malnutrition upon the physiological atrophy of the organ which begins shortly before puberty in the normal albino rat.

The higher degree of splenic atrophy in the males is in agreement with the fact that in normal 100 g. rats the ratio spleen/body weight is higher in females than in males.

The atrophy of the thyroid though relatively slight, is, nevertheless, significant in view of the rapid growth of the gland in the control animals. Thyroid atrophy may account to a great extent for the decline in metabolic rate observed during inanition.

The response of the adrenal gland to chronic inanition differs markedly from its reaction towards acute inanition. In the first condition the gland undergoes slight atrophy, as shown in our experiments and by Jackson (1915, 1919), McLennan & Jackson (1933), Mulinos & Pomerantz (1940,1941*a*), Mulinos, Pomerantz & Lojkin

(1942) and others, while after acute inanition there is enlargement of the adrenals, especially marked in the cortex, as reported by Andersen (1935), Stammers (1926), Cameron & Carmichael (1946) and others. This increase in weight is probably due to an alarm reaction—Selye (1936, 1937, 1946). Selye refers to malnutrition as a cause favouring the occurrence of alarm reaction induced by cold, over exertion, etc., but more recently he has shown in collaboration with Moya, Prado, Rodriguez, Savard & Selye (1948) that adrenal-cortical stimulation elicited by stress or unilateral adrenalectomy is greater under high than under low protein diets. The effect does not take place in hypophysectomized animals and it seems to be due to increased corticotrophin production.

When body weight is maintained constant by moderate underfeeding (diet 3) the adrenal weight also remains constant, a fact which has been observed by Guestalla & Rogoletti (1936). The comparatively greater atrophy of the adrenals among the females (diets 1 and 2) is noteworthy, since the adrenals are known to be larger in the female than in the male rat under normal conditions.

The adrenals show a resistance to inanition, second only to the *testes* which are the most resistant organs to the effects of malnutrition in the pubertal stage. It is interesting that both these organs are steroid producers.

The resistance of the testes is also shared, though in a lesser degree, by the organs dependent on its secretion, i.e. prostate, seminal vesicles and coagulating glands. Testicular atrophy is also difficult to obtain by underfeeding in ages other than the pubertal stage (Stewart, 1919; Siperstein, 1921; and others). The fact that female sex organs are more affected than male sex organs has also been observed by Jackson (1932).

The main differences in organ weight found between chronic inanition and hypophysectomy are:

(1) The marked thymus atrophy caused by restricted diet as opposed to the slight atrophy shown after pituitary ablation. This suggests that the presence of the pituitary might be necessary for the inhibition of thymus growth, which is effected through the gonads as shown by Evans & Simpson (1934), Chiodi (1938) and others, and through the adrenals as shown by Rapela (1944) and by Dougherty & White (1943). Ingle, Prestrud, Choh Hao Li & Evans (1947) obtained in rats fed with high calorie diets almost complete thymus atrophy after repeated injections of adrenocorticotrophic hormone. The effect was shown to be independent of the composition of the diet.

(2) The adrenals and gonads are severely affected by hypophysectomy while they are markedly resistant to inanition. Even after forced feeding, the weight of these organs in hypophysectomized animals is below that of underfed rats whose body weight is far lower.*

(3) The thyroid is also more affected by hypophysectomy than by the severest chronic malnutrition. Moreover, in the males forced feeding fails to lead to resumption of growth.

* Mulinos & Pomerantz (1940) could not obtain such a marked testicular atrophy with underfeeding as they did after hypophysectomy and they attributed this to the fact that the testes remained in the scrotum during inanition, while they ascended into the abdomen after hypophysectomy. This may well be the consequence, and not the cause, of the greater weight loss found in the latter condition.

(4) Kidney atrophy is greater after hypophysectomy than after underfeeding pointing to a regulating influence of the pituitary on kidney growth.

According to Mulinos & Pomerantz (1941*b*) and Pomerantz & Mulinos (1943) pituitary replacement partially restores some of the effects of inanition; body weight increases, the adrenal glands recover their normal weight, the gonads recover partially and the thyroid weight is very slightly if at all modified.

These results suggest a pituitary hypofunction during inanition, but they do not necessarily imply that this hypofunction is the primary state whereby the atrophy of the rest of the organism is caused, and such generalizations as the term 'pseudo-hypophysectomy' can be misleading, in view of the gross differences in organ weight found under both conditions.

It has been shown previously—Rinaldini (1949)—that the gonadotrophic potency of the pituitary gland is markedly increased in underfed rats, indicating that the atrophy of the ovaries and the anoestrus of chronic inanition, is not due to decreased production but rather to diminished release of pituitary hormones.

SUMMARY AND CONCLUSIONS

1. Organ and total body weight of 100 g. male and female albino rats underfed for 30 days are compared with those of normally fed rats, hypophysectomized rats and hypophysectomized rats submitted to forced feeding.

2. With the more restricted diets there was a loss of approx. 40 % body weight and with *ad lib.* feeding an increase of about 60 %. Thirty days after total hypophysectomy average body weight decrease was approx. 15 %.

3. The thymus is the organ that undergoes greatest atrophy during chronic inanition, while it is only slightly modified by hypophysectomy.

4. The gonads, notably the testes, are the most resistant to underfeeding. Adrenal glands are but slightly atrophied. On the other hand, these are the organs that undergo greatest atrophy after hypophysectomy. Kidney atrophy is also more marked in hypophysectomized animals.

5. In spite of the similarities that exist between chronic inanition and hypophysectomy, the above findings do not encourage the view that the influence of inanition on organ weight is exerted primarily through inhibition of pituitary secretion, even if there is some evidence of pituitary hypofunction.

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THE GRADIENT OF MUCOSAL SURFACE AREA IN THE SMALL INTESTINE OF THE RAT

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INTRODUCTION

In order to interpret gradients of absorptive activity in the small intestine of the rat, we recently required to know the nature of the gradient of mucosal surface area per cm. length of intestine. Whilst there is evidence for the existence of gradients of mucosal area per cm. length along the small intestine of the pigeon (Verzár & McDougall, 1936) and of the dog (Warren, 1939), the only data for the rat seem to be those of Wood (1944), which provide mean values of mucosal surface area per cm. for preparations of jejunum and ileum, the exact location of the segments measured being unspecified. Wood's data indicate a higher surface area per unit length in the jejunum than in the ileum, but in the absence of measures of location, they cannot be used safely to give any quantitative description of the gradient.

In the present work it was desired to measure the mucosal surface area in the conditions in which we have recently examined absorptive activity in the surviving isolated intestine (Fisher & Parsons, 1949*a*, *b*), namely when the intestine is distended mildly, by a pressure of 35 cm. H_2O.

In the intestine fixed under these conditions there are regions of flat mucosa between the villi such as can be seen when the intestine is normally distended with chyme (Johnson, 1913). The estimation of intestinal mucosal area by the method of counting villi and measuring the average area of a villus, used by early workers and by Verzár & McDougall (1936), is evidently not applicable in this instance. Warren (1939), after reviewing the early literature, gives an expression for the mucosal area as a simple additive function of the ratios of mucosal length to serosal length and of serosal circumference to mucosal circumference, the ratios being determined experimentally by measurements of longitudinal and transverse sections. The equation given by Warren was used by him to obtain values for the surface area of the dog's intestine at different levels, and by Wood (1944) to estimate the surface area of the rat's intestine. Warren does not justify in detail his expression for estimating surface area, but a derivation which we have made from first principles has shown that it is applicable only when the whole of the mucosa is folded into closely packed villi with negligible amounts of intervening flat mucosa: it is, in fact, a special case of a general expression which we give in the next section.

The modified Warren formula

The formula as given by Warren is

$$MA/SA = MC/SC + ML/SL - 1,$$

the symbols referring to the mucosal/serosal ratios of area, circumference, and length in the sections measured. For convenience we have replaced these symbols by R_a, R_c and R_l respectively.

Other symbols required are:

n_l = no. of villi per unit serosal length (long. sect.).

n_c = no. of villi per unit serosal circumference (trans. sect.).

f_l = fraction of mucosal length *not* occupied by villi.

f_c = fraction of mucosal circumference *not* occupied by villi.

Now take as a model of the mucosa a plain surface studded with rectangular prisms with bases of breadth b, and width w, and with heights h. Then, the mean area of one villus, excluding the top, equals $2h\,(b+w)$.

Since the number of villi per unit serosal area is $n_c n_l$, and since the area of the mucosa overlying unit serosal area is equal to the serosal area plus the area of the sides of the villi (the areas of the tops of the villi are equal to the areas lacking mucosa on which the bases of the villi stand), this gives mean mucosal area on unit serosal area (R_a) to equal

$$2h\,(b+w)\,n_c n_l, \quad \text{or} \quad R_a = 2hbn_c n_l + 2hwn_c n_l + 1. \tag{i}$$

In order to put this equation in Warren's form, h, b, w, n_c and n_l require to be expressed in terms of R_c and R_l so far as possible. We thus have it that the mucosal length per unit serosal length is

$$R_l = 1 + 2hn_l, \tag{ii}$$

and similarly that,

$$R_c = 1 + 2hn_c. \tag{iii}$$

Further, we can write,

$$f_l = 1 - wn_l, \tag{iv}$$

and

$$f_c = 1 - bn_c. \tag{v}$$

From equations (ii) and (v),

$$2hbn_c n_l = (R_l - 1)\,(1 - f_c). \tag{vi}$$

From equations (iii) and (iv),

$$2hwn_c n_l = (R_c - 1)\,(1 - f_l). \tag{vii}$$

Substituting from equations (vi) and (vii) in equation (i),

$$R_a = (R_l - 1)\,(1 - f_c) + (R_c - 1)\,(1 - f_l) + 1. \tag{viii}$$

In the special circumstances that the intestine is so far contracted that f_c and f_l are negligibly small, this reduces to $R_a = R_c + R_l - 1$, which is Warren's formula in our symbols.

Since the expression $(b+w)$, which is the measure of the perimeter of the base of a villus in this model, is eliminated from the final expression, it would seem that the form assumed for the cross-section of a villus does not influence the answer.

We have found, in fact, that equation (viii) holds both for a model with cylindrical villi and for a model with elliptical villi having the major axes of the bases parallel to one another, if the villi have flat tops and are randomly distributed about the plane of section. Equation (viii) is no longer exact for hemispherical villi randomly distributed about the plane of section. It yields an underestimate, but this is not likely to be less than 97 % of the true value even in the least favourable instance. For villi of the form of Fig. 1, which approximates more closely than any other of these models to the form of the rat's villus (which is lamelliform), equation (viii) is correct, if the villi are randomly distributed about the plane of section.

For a round-topped cylindrical villus the formula will fit with an exactitude intermediate between that for the cylinder and that for the hemisphere. Thus we may safely take the formula as a useful general relation.

METHODS

The whole small intestine of a male white rat (Wistar strain: body weight 200–300 g.) was set up on circulation units as described by Fisher & Parsons (1949*a*). Three units were used, the segments set up corresponding approximately to duodenum, jejunum and ileum. In this technique 50 ml. of oxygenated glucose-Ringer are circulated through the lumen of the intestine (inner fluid) and the intestine is bathed in another 50 ml. of well mixed, oxygenated glucose-Ringer (outer fluid). In these experiments half the inner fluid and half the outer fluid were replaced simultaneously by Bouin's fluid (Carleton & Leach, 1938, p. 30) as soon as the segment was set up, and the fixative was kept circulating for 30 min.

After the initial period of fixation on the circulation units, the segments were removed and measured. Two adjacent 1 cm. specimens were cut out at measured intervals beginning at or near the ileo-caecal valve. The specimens were then placed in Bouin's fluid for a further period of 24 hr. Routine dehydration, clearing and paraffin embedding followed. One of the two blocks taken from each level was used

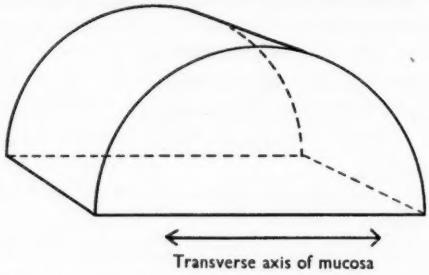

Fig. 1. Approximate model of the form of a villus in the rat's small intestine.

for cutting transverse sections and the other for cutting longitudinal sections. No block was made from any specimen containing a Peyer's patch. Two slides were made from each block, the sets of sections on each slide being separated by some 0·5 mm. Sections were cut at a thickness of 8μ. In practice less than 1 cm. of gut length separated the transverse and longitudinal sections at any level. The sections were stained with haematoxylin and eosin. Tracings were made by projecting with a micro-projector at a linear magnification of about 50 on to the back of a glass screen covered with 'Ethulon' plastic tracing film (May and Baker). One tracing was made from each slide so that two transverse and two longitudinal tracings were available for measurement at each level.

The measurements required were made on the tracings with a map measurer calibrated in cm. In addition to mucosal and serosal outlines on longitudinal and transverse tracings an additional measurement on each tracing was required for the computation of the factors f_c and f_l. This was obtained as shown in fig. 2, which represents a longitudinal section. Intervals corresponding to the lengths of mucosa not occupied by villi were marked off on the serosa, and the sum of the corresponding serosal intervals (the heavy lines in Fig. 2) was measured. This sum of serosal projections of lengths of mucosa was denoted by N_e and the corresponding sum for

the longitudinal tracing by N_l. Denoting the remaining measurements by M_c for mucosal circumference, S_c for serosal circumference, M_l for mucosal length, and S_l for serosal length, we have

$$\frac{M_c}{S_c}=R_c, \quad \frac{N_c}{S_c}=f_c, \quad \frac{M_c}{S_l}=R_l, \quad \frac{N_l}{S_l}=f_l. \tag{ix}$$

Substituting these expressions in equation (viii) we get

$$R_a=\frac{(M_l-S_l)(S_c-N_c)+(M_c-S_c)(S_l-N_l)}{S_cS_l}+1. \tag{x}$$

Since the individual measurements enter into the computation only in the form of the ratios (equation ix), the magnification factor is immaterial up to this point, and we take the symbols used to denote measurements made on the common but arbitrary scale of the tracings. It is only in deriving M_a, the mucosal area per unit serosal length, that the magnification factor, g, need be taken into account. If g be

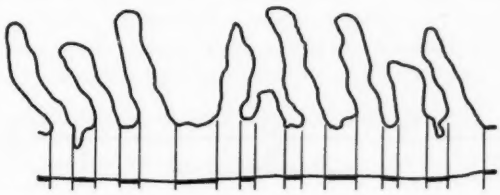

Fig. 2. Outline tracing of longitudinal section of rat's small intestine, to show the method of measuring N_l. For explanation see text.

cm. true length per cm. tracing length, then the true serosal surface area per unit serosal length is gS_c, and therefore

$$M_a=gS_cR_a. \tag{xi}$$

RESULTS

Detailed measurements on one animal at five different levels are shown in Table 1. It will be seen that there is a systematic increase in the values of mucosal area per unit serosal length in passing from the ileo-caecal valve towards the pylorus. The other point of interest concerns the magnitude of the values f_c and f_l, both of which are of

Table 1. *Estimates, at different levels in the intestine of a rat, of the parameters necessary for the determination of the mucosal surface area*

Location, cm. from ileo-caecal valve	R_c	R_l	f_c	f_l	R_a	Serosal circum. (cm.)	M_a (sq.cm./cm.)
80	2·65	3·27	0·478	0·340	3·27	1·73	**5·65**
60	2·16	3·17	0·619	0·410	2·67	1·87	**4·99**
40	2·12	3·13	0·635	0·454	2·39	1·87	**4·47**
20	2·23	2·84	0·661	0·435	2·31	1·88	**4·34**
10	2·01	2·85	0·624	0·515	2·17	1·76	**3·82**

(The values of the mucosal surface area per unit serosal length of the embedded specimens (obtained from equation (viii)) are shown in black in the right-hand column. Each value given is the mean of the results obtained independently from two sets of tracings as described in the text.)

the order of 0·5 in our preparations and therefore cannot be neglected. Fig. 3 shows a plot of estimates of M_a against distance in cm. from the ileo-caecal valve.

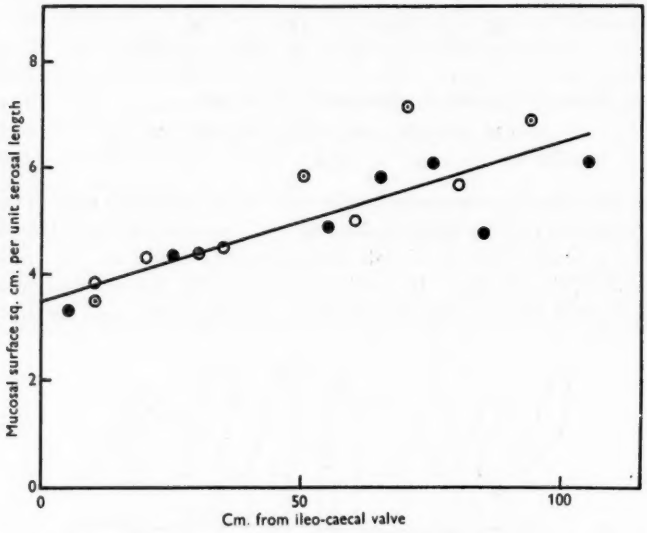

Fig. 3. Estimates of mucosal surface at various points of the embedded intestines of three rats and the best fit line relating the points. ○ = rat A; ● = rat B; ⊙ = rat C. The parameters of the embedded mucosal surface gradient have been calculated separately for each of these animals and are given in Table 2.

Table 2. *Characteristics of the mucosal surface area gradient in the embedded material from the three experimental rats*

Rat	Body weight (g.)	Fixed length of intestine	Total mucosal area (sq.cm.)	Mucosal surface area sq.cm. per cm. at point 50 cm. from ileo-caecal valve	Mucosal area gradient		
					Intercept* (a)	Slope† (b)	n'
A	275	97·5	470	4·85±0·07	3·66	0·0237	(5)
B	280	115·0	609	5·11±0·22	3·86	0·0251	(7)
C	325	95·5	516	5·52±0·29	3·24	0·0455	(5)
Combined data				5·05±0·16	3·53	0·0302	(17)

* The intercept parameter, a, is the mucosal surface area, sq.cm. per cm. serosal length, at the ileo-caecal valve.

† The slope, b, of the gradient implies that in animal A, for example, the mucosal surface area per cm. of serosa increases by 0·0237 sq.cm. per cm. increase of distance from the ileo-caecal valve.

n' signifies the number of pairs of observations used to compute the equation of the surface area gradient.

The data from all three animals are plotted and each point is the mean of estimates derived independently from the two sets of tracings made from each block of tissue. The points on Fig. 3 lie reasonably well about a straight line, and there is clearly no support for any more elaborate relation than a linear one between M_a and distance from the ileo-caecal valve. The best fitting line has the equation

$$M_a = 0{\cdot}0302x + 3{\cdot}53, \qquad \text{(xii)}$$

where M_a is the mucosal surface area per cm. serosal length of the embedded specimens at a distance x cm. from the ileo-caecal valve measured after the initial fixation.

Values for the regression parameters for the individual animals are given in Table 2, together with the estimates obtained for the total mucosal surface area of the embedded intestine.

Systematic errors

We have investigated the extent of shrinkage of the small intestine during the initial fixation by comparing the total lengths of small intestines immediately after removal from the circulation units with the total lengths of unfixed small intestines which we have measured regularly in our studies of absorption from the surviving intestine (Fisher & Parsons, 1949*a*, *b*). Measurements of fifty unfixed intestines of male rats, taken at random from our records, were classified by body weight, and the mean intestinal length in each group was computed (Table 3). It will be seen that there is no appreciable variation in intestinal length over a wide range of animal weights (158–415 g.). Our fixed material, prepared as already described, comprises six whole small intestines, the lengths of which, determined after the initial fixation on the circulation units, are given in Table 4. The mean length, 101·1 cm. differs from the mean length of the unfixed specimens by $10{\cdot}1 \pm 3{\cdot}5$ cm., and is therefore significantly less. We have therefore adopted a correction factor of $\frac{111{\cdot}2}{101{\cdot}1} = 1{\cdot}10$ to correct the values of x in equation (xii) for shrinkage during the initial fixation.

Table 3. *Body weights and unfixed lengths of small intestine of fifty randomly selected male white rats*

No. of animals	Body weight range (g.)	Mean body weight (g.)	Mean intestinal length (cm.)
11	158–199	181·0	$107{\cdot}8 \pm 1{\cdot}925$
12	200–249	223·3	$111{\cdot}8 \pm 2{\cdot}814$
15	250–299	273·7	$111{\cdot}9 \pm 2{\cdot}245$
12	300–415	334·3	$112{\cdot}9 \pm 2{\cdot}138$
All groups: 50	158–415	255·8	$111{\cdot}2 \pm 1{\cdot}159$

Table 4. *Body weight and total length of small intestine of male rats, the measurements of length being made after the initial period of fixation in Bouin's fluid on the perfusion units*

	Body weight (g.)	Length of fixed intestine (cm.)
	275	97·5
	280	115·0
	325	95·5
	250	106·0
	227	96·5
	200	96·0
Means	259·5	$101{\cdot}1 \pm 3{\cdot}21$

If it is assumed that shrinkage occurs equally in all directions, then it will operate as a systematic error in the magnification factor applicable to all measurements made on the preparations, and we have already shown that this will be without effect on

the estimation of R_a. Referring back to equation (xi), the estimate of M_a will therefore be affected by shrinkage only so far as shrinkage affects S_c. We shall therefore have

$$M'_a = 1{\cdot}10\, M_a, \quad x' = 1{\cdot}10\, x,$$

where M'_a and x' are the values corrected for fixation shrinkage. In these terms equation (xii) becomes

$$M'_a = 0{\cdot}0302\, x' + 3{\cdot}88. \tag{xiii}$$

The stages subsequent to the initial fixation, including embedding, result in further shrinkage of the material, which we have measured in the following manner.

Immediately after fixation on a circulation unit a 5 cm. length of intestine was taken and divided transversely into three approximately equal lengths. The upper and lower portions were taken through to the paraffin embedding stage. The middle portion was used to measure serosal circumference in the freshly fixed state. To make this measurement thin transverse rings were cut from the freshly fixed tissue. Each ring was then cut open and laid out, mucosal side down, as a narrow strip on a microscope slide. There was no wrinkling of the serosal coat, and it was possible to tell by inspection of the form of the strip edges whether the rings were truly transverse sections. Truly transverse rings gave straight strips; rings deviating at all appreciably from transverse formed angulated strips. The length of satisfactory transverse strips was measured on the scale of a mechanical stage under the microscope, using an eyepiece micrometer as an aid to alinement. The mean estimate of this length was taken as the serosal circumference in the freshly fixed state.

The corresponding measurements on embedded material were made by tracing outlines of projections of mounted sections at a magnification of *c.* 50, and measuring serosal circumference with a map measurer. Typical measurements of serosal circumference are: freshly fixed, $1{\cdot}93 \pm 0{\cdot}008$ cm. (7 obs.); embedded, $1{\cdot}70 \pm 0{\cdot}006$ cm. (4 obs.).

This gives a further correction factor of 1·13 $\left(=\frac{1{\cdot}93}{1{\cdot}70}\right)$, but as the shrinkage to which this refers occurs subsequent to the measurement of intestinal length, the correction is applicable only to M'_a. The fully corrected equation therefore is

$$M''_a = 0{\cdot}0341\, x' + 4{\cdot}38, \tag{xiv}$$

and in this form provides an estimate of the mucosal surface area in sq. cm. per cm. serosal length at any point in the unfixed intestine distant x' cm. from the ileo-caecal valve.

Table 5 summarizes our findings with respect to the effects of shrinkage on mucosal surface area.

Table 5. *Summary of estimates of mucosal surface area of rat's small intestine*

	Total mucosal area (sq.cm.)	Mucosal surface area, sq.cm. per cm. serosal length			Estimated distance pylorus-ileo-caecal valve (cm.)	Mean surface area per unit serosal area (sq.cm.)
		At ileo-caecal valve	At mid-point of intestine	Immediately below pylorus		
Embedded tissue	500	$3{\cdot}53 \pm 0{\cdot}642$	$5{\cdot}06 \pm 0{\cdot}160$	$6{\cdot}59 \pm 0{\cdot}642$	101·1	3·7
Fresh tissue	700	$4{\cdot}38 \pm 0{\cdot}798$	$6{\cdot}28 \pm 0{\cdot}234$	$8{\cdot}17 \pm 0{\cdot}798$	111·2	

DISCUSSION

Comparison with other estimates of surface area of rat's small intestine

It will be seen from fig. 3 that on average the mucosal area per cm. serosal length of the embedded preparations at a point 50 cm. from the ileo-caecal valve is about 5·0 sq.cm. (This represents almost exactly the mucosal area per cm. at the mid-point of the small intestine.) Wood's (1944) estimate for the same location and for embedded material is 6·8 sq. cm. per cm. length. It thus appears at first sight that our figures are systematically lower than hers. But she used the original Warren expression, and if this expression is applied to our measurements, it gives an estimate higher than hers, namely 7·9 sq.cm. per cm. length at 50 cm. from the ileo-caecal valve.

Thus the difference between Wood's estimate and ours is less than the error involved in applying the Warren expression to our material. This suggests that the difference between the two sets of observations may be no more fundamental than a difference in degree of distension of the specimens.

On the other hand, our estimates differ from those of Verzár & McDougall (1936; p. 9) both with respect to the length of the intestine, which they put at 60 cm. against our figure of 111 cm., and with respect to the total mucosal surface which they estimate as 450 sq.cm., against our estimate of 700 sq.cm. The length discrepancy strongly suggests failure to correct for shrinkage. But such a failure would still not suffice to account for the area discrepancy.

Effects of intestinal distension on estimates of mucosal surface

For our purpose, i.e. for the estimation of surface area in the conditions of our observations of the absorptive activities of surviving intestine, distension need not be considered as a source of systematic error: our measurements of surface area have been made at the distension pressure used in our studies of absorptive activity. But the observations would be of greater general use if it could be taken that degree of distension had no effect on the estimate of mucosal area.

Warren (1939) has implied that distension may alter the mucosal surface area, but it is difficult to see how this can happen as a result of stretching of the whole mucosal sheet unless the degree of distension is such that the serosa is stretched until its surface is of the order of that of the mucosa. Only at this point can distension cause stretching of the whole mucosa. On the other hand, it is certain that distension does stretch the serosa sufficiently to cause a degree of unfolding of the mucosa. If this unfolding involves the exposure of mucosa previously counted as within the crypts of Lieberkühn, and, as such, excluded from the estimate of mucosal surface, then distension will increase mucosal surface. However, sections of crypts in the appropriate planes indicate that in the rat they are cylindrical in form. Such cylinders could only be evaginated appreciably into the general mucosal surface by stretching of the mucosal sheet. We take it as extremely unlikely that such stretching occurs, both for the reason already given and since it would involve the distortion of the individual cells in the manner observed by Johnson (1913). Johnson distended the intestines of guinea-pigs at pressures of from 150 to 270 cm. of water and observed considerable flattening of the mucosal cells of both the large and small

intestine. His figs. 10 and 11 represent the appearances for the mucosal cells of guinea-pig's colon at different distension pressures, and he states that similar pictures may be obtained for the small intestine. Fisher & Parsons (1949*a*; Fig. 2) show the appearances of the mucosa of the small intestine of the rat at distension pressures of 0, 10 and 35 cm. of water. Neither these illustrations nor any of the many microscopical preparations we have made reveal any flattening of the cells in the mucosa in the region of the crypts. We have therefore concluded that in the rat there is trivial unfolding of crypt mucosa over a distension pressure range of 0–35 cm. of water, and consequently our estimates of the mucosal surface are applicable to rat intestines fixed at distension pressures within this range.

Application of the anatomical gradient of surface area to physiological gradients in the rat's intestine

The relation between M''_a (the fresh intestine) and location described in equation (xiv) may be used directly to estimate the surface area per unit length of a segment of small intestine of any length, provided that the distance of the centre of this segment (the 'mean distance') from the ileo-caecal valve is known. Where there is a linear relation between mucosal area and distance from ileo-caecal valve the mean value for any segment of the area per cm. length must coincide with the area per cm. at the mid-point of the segment, so that we may write:

$$A/l = 0{\cdot}0341\,m + 4{\cdot}38, \qquad \text{(xv)}$$

where A/l is the average mucosal area per cm. length of a segment of length l whose centre point is distant m cm. from the ileo-caecal valve. In this form the relation can be used to test directly whether the ability of the intestine to absorb or metabolize a particular substance is one shared by all cells of the mucosa equally, or whether one must postulate a multiplicity of cell types in the mucosa. If the ratio of absorptive activity per unit length to mucosal area per unit length is sensibly constant there is no necessity to postulate more than one type of cell (though indeed the activity measured could be due to a type of cell forming a constant fraction of the mucosal population). On the other hand, if the activity per unit mucosal area changes largely as one passes along the intestine, at least two cell types must be postulated.

With the aid of the present observations it is now possible to compute from the data of Fisher & Parsons (1949*b*) the glucose utilization per unit mucosal area at different locations in the intestine, and also the rate of transfer of glucose per unit mucosal area across the mucosa, both measurements being made in standard conditions of glucose concentration. Fig. 4 illustrates these relations. The utilization per unit mucosal area is almost constant throughout the intestine, but the rate of glucose transfer across the mucosa increases rapidly as the distance from the ileo-caecal valve increases. We have previously shown (Fisher & Parsons, 1949*b*) that the gradient of glucose translocation per cm. length of intestine is significantly different from that of glucose utilization (i.e. that the one relation could not be obtained within the limits of experimental error from the other by multiplication by an appropriate factor). The new finding here is that the gradient of glucose utilization is explicable on the simple hypothesis that glucose utilization is proportional to mucosal surface area. This suggests that the mucosa is responsible for most of the

intestinal glucose utilization and that the mucosal cells are approximately equipotent in capacity to utilize glucose.

One cannot escape the conclusion, however, that all mucosal cells are not equally active in the secretion of glucose into the tissue fluids. There must be at least two types of mucosal cell with different activities in this respect. There are not adequate data for the distribution of cell types in the mucosa to indicate which cell type is principally concerned in glucose translocation, but in the light of these studies it seems probable that quantitative cytological investigation of the small intestine might now throw light on the activities of the different types of mucosal cell.

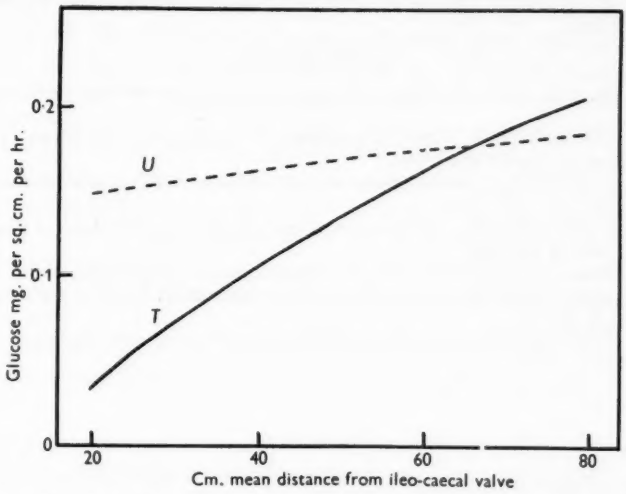

Fig. 4. Relation, for rat's intestine, between glucose utilization, U, glucose translocation across mucosal cells, T, and loop mean distance. The data, recalculated from Fisher & Parsons (1949*b*), applies to loops exposed to an initial concentration of glucose in the lumen of 0·5%. For further explanation see text.

SUMMARY

1. A new expression has been derived relating mucosal surface area to linear measurements of transverse and longitudinal sections of intestine. Differences in villus conformation are shown to have trivial effect on the accuracy of the area estimates so obtained. The new expression can therefore be applied to measurements made on any species, and this paper describes its application to the rat's small intestine.

2. The extent of shrinkage during histological procedures has been investigated, and appropriate corrections for it are described.

3. The mucosal area per cm. length of small intestine is shown to increase linearly with distance from the ileo-caecal valve. After correction for shrinkage the estimates are: mucosal area at 100 cm. from the ileo-caecal valve, 8·17 sq.cm. per cm. length. The mean value for the whole small intestine is 6·3 sq.cm. per cm. length.

4. For male rats of the strain used, the length of the small intestine is independent of body weight over 150 g., and has a mean value of 111 cm. The corresponding estimate of total mucosal surface is 700 sq.cm.

5. The linear gradient of mucosal surface is discussed in relation to recently described gradients of glucose utilization and of active translocation of glucose across the mucosa. Glucose utilization is nearly exactly proportional to mucosal surface. Glucose translocation per unit mucosal surface increases steeply from the ileo-caecal valve, and it is suggested that quantitative cytological study might indicate which of the mucosal cell types could be responsible for this process.

We wish to thank F. C. Caddick and Olive L. Drage for skilled assistance in preparing histological specimens and for help with the measurements. We are grateful to Prof. R. A. Peters for his interest and encouragement and to Mr E. H. Leach for help and advice.

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THE INTRAMURAL BLOOD SUPPLY OF THE UPPER JEJUNUM IN MAN

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Jonnesco & Soulié (1912), in their account of the intramural blood supply of the jejunum, describe the vasa recta of the bowel as dividing into two equal branches which encircle the gut to anastomose with each other across the anti-mesenteric border. They describe a rich arterial plexus in the submucosa from which small branches are given off to supply the mucous membrane, and they give an account of the vascular arrangement of a typical villus. Similar accounts are given by Gérard (1912), Symington (1914), Drennan (1943) and Mottershead (1946), but a fuller description is given by Davies (1946). Greater detail is provided, however, by Eisberg (1924, 1925), Cokkinis (1930), and Latarjet (1931), the last named having made extensive use of radiological technique. The most recent account is that of Noer (1943), who injected the vessels of the bowel wall with latex rubber solution, later clearing them by the Spalteholz method. Experimental surgery has also contributed to the subject, particularly with regard to the question of intramural anastomoses. The rapid development of the radiological technique in recent years, however, suggested that a fresh study by these new methods might be of value.

METHOD

The material studied consisted of the first loop of the jejunum, with its mesentery, removed from twelve adult bodies at autopsy. Seven were males and five were females. The average length of the specimens removed was 15 in.

To demonstrate the arteries a suspension of 20 % bismuth oxychloride was used; for the arterioles and capillaries a 10 % colloidal silver iodide was employed (Barclay, 1947; Barclay & Bentley, 1949).

The injections were made with a syringe through a hypodermic needle tied into a convenient artery in the mesentery, the small bore of such a needle being a convenient safeguard against the development of too great a pressure leading to rupture of the smaller vessels. The cut ends of all the other arteries in the mesentery were ligatured to prevent leakage of the contrast medium, and the injection was continued till a slight resistance was encountered.

After the injection was completed the loop of bowel and its mesentery were radiographed to confirm the general satisfactory filling of the arterial tree. The bowel was then slit longitudinally just to one side of its mesenteric attachment, opened out flat, and again radiographed. This second film was examined in more detail with a lens, and areas of particular interest were selected for further study and micro-radiography.

THE INTRAMURAL DISTRIBUTION OF THE VASA RECTA

The observations of Eisberg (1924) and Cokkinis (1930) that, on reaching the mesenteric border of the jejunum, the vasa recta pass alternatively on either side of the bowel were confirmed. The vessels were not found to divide into two branches encircling the gut in any of the specimens examined, as has been frequently described.

The majority of the vasa recta arborize on the bowel wall as they run towards the anti-mesenteric border; but some continue for a considerable distance before branching (Pl. 1, figs. 1 and 2). It will be noted in these radiographs that small vessels are given off from both sides of the parent vessel, and also from its branches both before and after the main bifurcation. The direction of these small vessels is variable, but they run more in the long axis of the bowel than do the larger ones; and some of these small vessels, near the mesenteric border, recur towards the mesentery. The main branches arising from the primary bifurcation of the vasa recta pass to the anti-mesenteric border where their bulk decreases rapidly and they end, usually, in two twigs. These, in turn, give off smaller and smaller branches until the very small terminal arterioles leading into the arterial side of the capillary bed are reached.

THE RELATIONSHIP OF THE ARTERIOLES AND THEIR BRANCHES TO THE CIRCULAR FOLDS OF THE MUCOSA

In certain specimens, such as that shown in Pl. 1, fig. 1, it was noticed that the injection mass filling the arterioles appeared to be concentrated in the circular folds of the mucous membrane. Moreover, it was seen, with a lens, that the leashes of arterioles tended to be arranged in parallel rows separated by relatively avascular areas. To determine the arrangement more precisely one of the circular folds was cut off, laid flat on its side, and a micro-radiograph was made. The result, shown in Pl. 1, fig. 4, confirms the fact that the parallel leashes of arterioles do lie within the mucosal circular folds.

RELATIVE AVASCULARITY OF THE ANTI-MESENTERIC BORDER

Reports concerning the vascularity of the anti-mesenteric border of the bowel are not in agreement. Eisberg (1924) states that the intramural arborizations of the vasa recta, when they reach the convexity of the intestinal loop, anastomose freely with similar branches from the arteries of the opposite side. Prof. Brash informs me that the same arrangement has been found by J. A. Ross (1947). Cokkinis (1930), however, claimed that the anti-mesenteric border was free from all visible vessels. Between these two views is that of Noer (1943), that this region is relatively avascular but that circumferential anastomoses do occur. This last opinion is the one which, with certain reservations, has been confirmed by the present study. Pl. 2, fig. 5, in which both the large and small vessels have been filled, shows a definite, but somewhat irregular, band running along the anti-mesenteric border which is of a lower vascular density than the rest of the specimen. Using the coarse injection material, which will not enter the smaller vessels, this avascular band becomes more striking Pl. 2, fig. 6); but, nevertheless, there are irregular areas where the anastomosis is

free (Pl. 3, fig. 7). In some specimens, however, and using the fine injection material, this avascular band is not present, showing that there is some variability in the anastomosis.

LONGITUDINAL ANASTOMOSES

Both Eisberg (1924) and Noer (1943) claim that there are numerous anastomoses between the smaller branches of adjacent vasa recta in the bowel wall, but Cokkinis (1930) states that there is no evidence to suggest the occurrence of anastomoses between adjacent arteries. The present investigation shows this latter view to be true only in regard to arteries of the calibre of the primary branches of the vasa recta on the bowel wall. This point is demonstrated clearly in Pl. 1, figs. 1, 2. It is not true of the smaller arteries, nor of those arising from the sides of the larger vessels. The position of these anastomoses is irregular and does not conform to any set pattern, but they do seem to be more numerous on the convexity than on the concavity of the intestinal loop. To elucidate the functional extent of this longitudinal anastomosis the ligation experiments of Eisberg (1924) and Cokkinis (1930) were repeated. The latter found that ligation of a contiguous group of vasa recta in the mesentery completely excluded the injection material (indian ink) from the part of the gut supplied by those vessels. Using the coarse injection fluid described above similar results can sometimes, but not invariably, be obtained (Pl. 3, fig. 8). In some specimens the fluid was seen to run through patent neighbouring vessels to the anti-mesenteric border and then back down the branches of the blocked artery to the ligature on the stem of the vasa recta in the mesentery. If the finer colloidal silver iodide is injected similar ligations of the vasa recta never exclude any part of the gut wall. Indeed, if the vasa recta in the mesentery are ligated over a length of 3 in. from the cut end of the specimen, so that the fluid can only enter through anastomoses from the opposite end, it will be found, with this finer material, that the vessels of nearly the whole loop can be filled. In other bodies the series of longitudinal anastomoses between the small branches is not so efficient. Pl. 3, fig. 9, shows a specimen, the excluded end of which, in spite of a satisfactory injection, has been little more than half filled. If the ligatures are placed in the centre of the loop, so that the excluded area can be supplied from both ends, then, of course, longer areas can be filled by way of the intramural anastomoses.

SUMMARY

1. Radiographs are shown to demonstrate the distribution of the vasa recta and their main branches in the bowel wall, and also that of the smaller arteries and arterioles which spring from the parent vessels.

2. It is demonstrated that the arterioles tend to be arranged in parallel rows lying inside the two mucosal leaves of each circular fold.

3. The relatively avascular nature of the anti-mesenteric border is confirmed, but is shown to be true of only a certain number of bodies. Radiographs are shown to demonstrate the circumferential anastomoses of the terminals of the vasa recta from opposite sides of the bowel.

4. Longitudinal anastomosis is shown to be poor and to depend upon small vessels. The efficiency of this system is demonstrated by radiographs following ligation of

the vasa recta. It is found to be variable, and at best to be able to bridge a gap of $2\frac{1}{2}$–3 in.

I wish to thank Prof. G. A. G. Mitchell for his help in the preparation of this paper. Also I wish to thank Mr F. L. Newell, Radiographer to the Department, for technical assistance. I am indebted to Mr Gooding and Mr Howarth for the photographs, and to Mr Pearson for the microradiographic sections.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. The arrangement of the main branches of the vasa recta on the bowel wall, with examples of the type that remains a single vessel without arborizations. (×1.)

Fig. 2. The arrangement of the main branches of the vasa recta on the bowel wall, with examples of the type that remains a single vessel without arborizations. (×1.)

Fig. 3. Demonstration of the tendency of the injection material to collect in the circular folds. (×1.)

Fig. 4. Microradiograph of a single circular fold lain upon its side to show that the arterioles are arranged in parallel rows within the two mucosal layers of that structure. (×12.)

Plate 2

Fig. 5. Showing the relatively avascular anti-mesenteric border as a band of lower vascular density running horizontally across the middle of the specimen. (×1.)

Fig. 6. A more striking demonstration of the lower vascular density of the anti-mesenteric border made by using a coarse injection material. (×1.)

Plate 3

Fig. 7. Demonstration of free circumferential anastomoses across the anti-mesenteric border. (×1.)

Fig. 8. Specimen injected with coarse material to show the bands of avascularity which may result from ligature of the vasa recta in the mesentery. (×1.)

Fig. 9. A specimen in which 3 in. of the bowel has been deprived of its direct arterial supply by ligatures on the vasa recta, and in which less than half of the excluded area has become filled. (×1.)

DORAN—Intramural blood supply of upper jejunum in man

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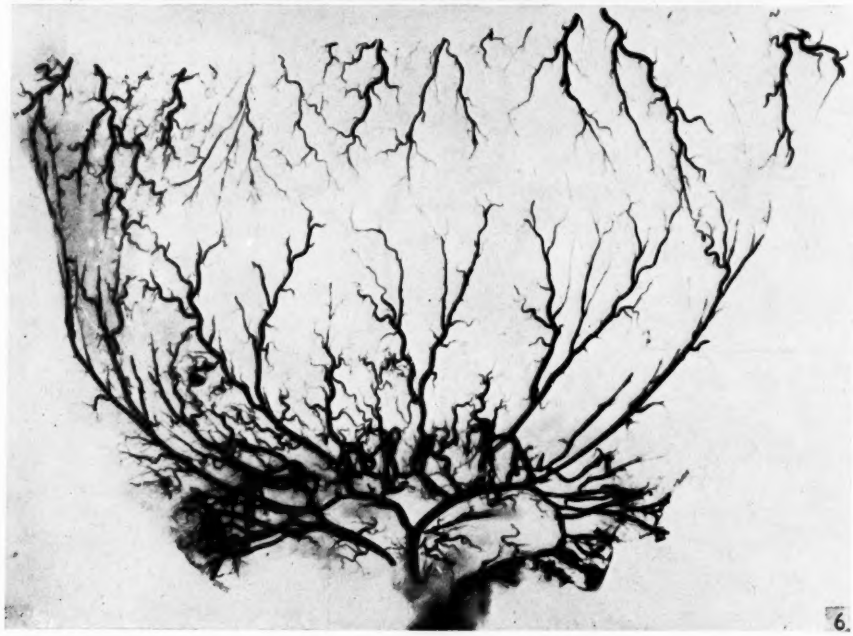
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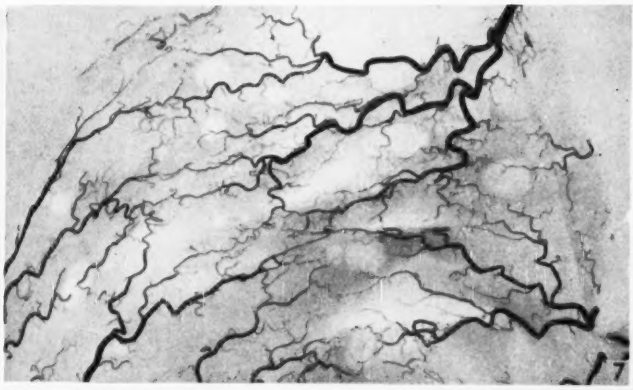
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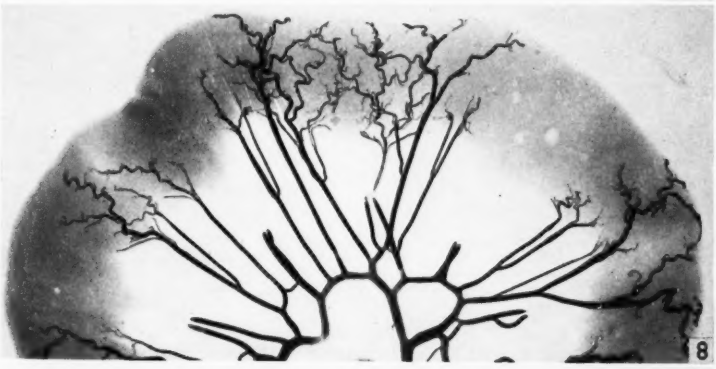
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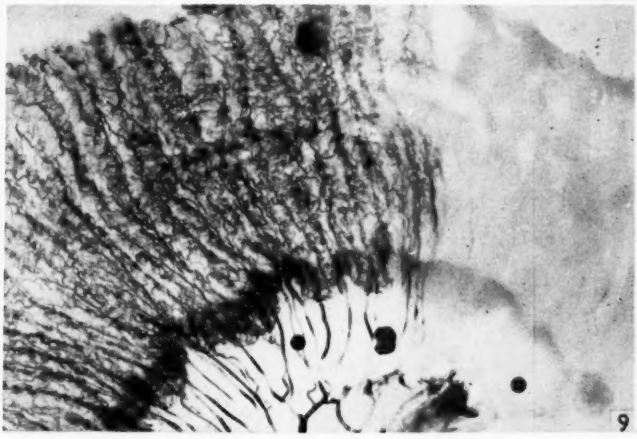
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THE DEVELOPMENT *IN VITRO* OF THE SUBMANDIBULAR AND SUBLINGUAL GLANDS OF *MUS MUSCULUS*

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INTRODUCTION

The tissue of the submandibular gland has been cultivated *in vitro* by several observers. Champy (1913) states briefly that he observed a rapid dedifferentiation in such explants, Nasu (1923) cultivated the submandibular gland of the adult rabbit and noted degeneration followed by epithelial regeneration. Mitsuda (1923), in cultures of similar material, saw the formation of solid sprouts from the excretory ducts; later the sprouts acquired a lumen by degeneration. Chlopin (1923) explanted the submandibular gland of rabbits 2 days after birth; he observed the formation of new acini which developed partly by cellular multiplication and partly by rearrangement of the ducts. The parotids of rabbits, aged from 3 days to 3 months, and of a human foetus, were grown *in vitro* by Zymbal (1932) who recorded the outgrowth of mesenchyme and epithelial laminae, and necrosis in the centre of the explant.

In the present study the salivary glands of the mouse were explanted at different embryonic stages and their development *in vitro* was investigated. The rudiments were found to differentiate in culture in a surprisingly normal way.

TECHNIQUE

Material

The embryos were removed aseptically from the uterus and decapitated. The head was immersed in Tyrode solution, and with the aid of a dissecting binocular microscope the brain was removed and the palate was split from above in the mid-line, the dorsal surface of the tongue thus being exposed. The rudiments of the submandibular glands were then seen as a pair of clear, oval structures just behind the free part of the tongue and in line with its lateral borders. In the later embryos the rudiment of the sublingual gland was also present close to that of the submandibular. The epithelial portion of the gland was surrounded by a dense, well-defined mass of connective tissue representing the capsule.

From most of the younger embryos only the submandibular gland and its capsule were removed for cultivation because the sublingual gland had not yet appeared. In the later stages in which both glands were present, the submandibular and sublingual rudiments were usually removed and explanted together; if the two rudiments were dissected apart and grown separately, the subsequent development of the sublingual and sometimes of the submandibular also, was irregular.

For the earlier stages the degree of development was estimated by the number of epithelial branches which could be readily counted provided they were not more than 10 in number. When development was more advanced, and the branches too numerous

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to count accurately, the stage of development was assessed by the gestation period, as calculated from the appearance of the copulation plug, and checked by the histological examination of one or more control glands in each litter. In the same embryo both glands are always at exactly the same stage of differentiation, but sometimes there is some variation between the glands of different members of the same litter.

Tissue culture

The rudiments were grown either by the hanging-drop or the watch-glass method (Fell & Robison, 1929).

For the hanging-drop preparations $\frac{7}{8}$ in. cover-slips were used. Some of the explants were grown on the surface of the medium, which consisted of equal parts of fowl plasma and 11–12-day chick embryo extract, and others were embedded in the clot in the usual way. This method was best for the cultivation of early rudiments (13th–14th day of gestation).

The older glands grew better in watch-glass cultures. They were explanted on the surface of a clot composed of four drops of plasma and four drops of embryo extract.

The rudiments were observed at least once a day, and while their structure was not too complex their outlines were drawn daily with the aid of a camera lucida. Explants maintained for more than 4 days were transferred to fresh medium on the 3rd or 4th day.

Histology

The explants were fixed at intervals of 1–7 days in Susa, Zenker's or Bouin's fluid. Some were stained with carmalum and mounted whole, but most were serially sectioned. Sections were stained with haematoxylin and eosin or azan.

To compare the development of the explants with that of the normal gland, normal rudiments were fixed at different stages ranging from the 13th day of gestation up to birth.

NORMAL DEVELOPMENT

The following brief account of the normal development of the submandibular and sublingual glands of the mouse is given to provide a standard of comparison for the development of glands in culture.

Stage 1. The submandibular gland first appears at about the 12th day of gestation as a tiny, spherical bud projecting inwards from the mouth epithelium. The adjacent connective tissue has not yet condensed to form the capsular rudiment.

Stage 2. Although usually found at the 13th day, this stage may also appear at the 14th and even at the 15th day. The submandibular bud is elongated and differentiated into two parts: a terminal swelling destined to form the adenomeres, and a small cord representing the rudiment of the main duct; the cord, which is solid, enters a pit in the floor of the mouth. The terminal bud also is solid and consists of a thick outer layer enclosing a central mass of cells. The peripheral cells, in which mitoses are very rare, are cylindrical and closely packed and their nuclei are situated in the middle of their long axes. The inner cells are irregular in shape, and are separated from one another by intercellular spaces; many of them are in division.

The epithelial bud is surrounded by a large well-defined mass of connective tissue which represents the capsular rudiment. The epithelial bud occupies only a small part of the capsular mass.

The early rudiment of the sublingual gland appears at this stage as a small bud.

Stage 3 (13th–15th day). The first sign of branching appears as a small cleft in the terminal bud.

Stage 4 (13th–15th day). Four to ten well-defined branches have now been formed in the submandibular gland and the sublingual has reached stage 2 and sometimes stage 3 of its development.

Some of the submandibular buds are attached directly to the rudiment of the main duct but others are connected by a thin neck which will become the intermediate tract. Both the buds and the duct rudiments are solid and composed of a regular outer layer of cylindrical cells enclosing an inner mass of loosely arranged cells of irregular form. The epithelium has now penetrated more deeply into the underlying connective tissue capsule, which encloses both glands and is very dense and distinct from the surrounding tissue.

Stage 5 (15th day). The submandibular gland has become richly branched and the sublingual has four to five buds. The connective tissue capsule is completely occupied by epithelial branches except for a small dome at its inner end. The submandibular buds are still solid but the cells of the outer layer are less compressed and cylindrical than before and their nuclei are now basally situated. The inner cell mass remains unchanged.

The intraglandular ducts are hollow. Their walls consist of an inner layer of cylindrical, deeply staining cells, and an outer layer of flat, less stainable cells. Continuity between the two layers of the ducts and the corresponding layers of the terminal buds are often demonstrable, but the lumen terminates at the junction between the duct and the acinar rudiment. The main duct is hollow in places, but the lumen does not seem to be continuous throughout its length.

The sublingual buds are solid; their duct may show the beginning of cavity-formation.

Stage 6 (16th day; Pl. 2, fig. 13). The acini of the submandibular gland are very abundant. Most of them are still solid and both mitosis and degeneration are seen in the cells especially in the inner mass. The lumen of the intermediate ducts has begun to penetrate into some of the acini and a few have already acquired a small cavity. Granular material can be seen inside the ducts.

Stage 7 (16th day; Pl. 3, fig. 15). More acini in the submandibular gland contain a central cavity which appears to be the definitive lumen; it is surrounded by several layers of cells, among which other intercellular spaces are seen which are not yet in communication with the central cavity. There is no sign of secretion. The main duct is now hollow throughout its length. Cell degeneration, though rather rare, is present in the acini and is frequent in the walls of the duct.

Stage 8 (17th day; Pl. 3, fig. 18). Nearly all the acini of the submandibular gland are hollow, and in the sublingual mucous secretion has begun.

DEVELOPMENT *IN VITRO*

I. *Rudiments at the single bud stage* (*stages* 1–3)

A few cultures were made of rudiments at stage 1, i.e. when the submandibular gland consisted of a tiny epithelial bud. After 24 hr. the bud differentiated into a cord with a terminal swelling (stage 2 of normal development), but it rarely showed any sign of branching and after 3 days regressed to form a small structureless mass of epithelium.

Fifty-five cultures were made at stages 2 and 3, when the submandibular anlage consisted of an epithelial cord (the rudiment of the main duct) with a terminal swelling which sometimes showed a cleft indicating the beginning of branching. Of these, eighteen explants were rejected as being degenerate or grossly abnormal and three were lost. The remaining thirty-four were observed and drawn daily, and were fixed at intervals of 2–6 days; thirteen were mounted whole and twenty-one were serially sectioned.

During the first few hours of cultivation the cord lengthened and became sharply distinct from the terminal bud. One or more notches appeared in the latter and deepened until the bud was subdivided into several branches. Each branch then formed a thin cord or neck by which it was united to the rudiment of the main duct. This process of subdivision was repeated so that more and more daughter buds were formed and the branching of the gland became increasingly complex. The process was the same as in normal development but advanced more slowly.

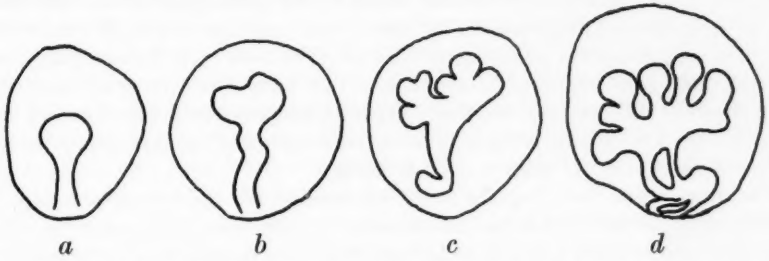
The necks of the buds to which new necks were continually being added, constituted the excretory portion of the gland connecting the acini with the main duct. At this stage the different parts of the excretory system—preterminal tubules of Heidenhain (1921), striped tubules of Zimmermann (1927), or salivary tubules of Pflüger (1886)—could not be distinguished, and all the epithelial structures between the acini and the main duct have therefore been termed intermediate tracts while solid, and intermediate ducts when they became hollow. The original cord acquired its cavity very quickly and has therefore been called the main duct from the beginning.

The number of branches formed varied in different cultures, so that in 3–5 days different explants might form anything from four to twenty primitive acini which were sometimes grouped in lobules. Text-figs. 1 and 2 show respectively examples of a modest development and of a comparatively rich one in two glands taken from litter-mates. The gland shown in Text-fig. 1 formed 6 buds and the cord acquired a small cavity. That drawn in Text-fig. 2 produced such a complicated tree during 4 days' cultivation that it was difficult to draw the outline with the camera lucida. Serial sections showed that the more richly branched glands had a correspondingly more advanced histological development than those which had formed fewer acini.

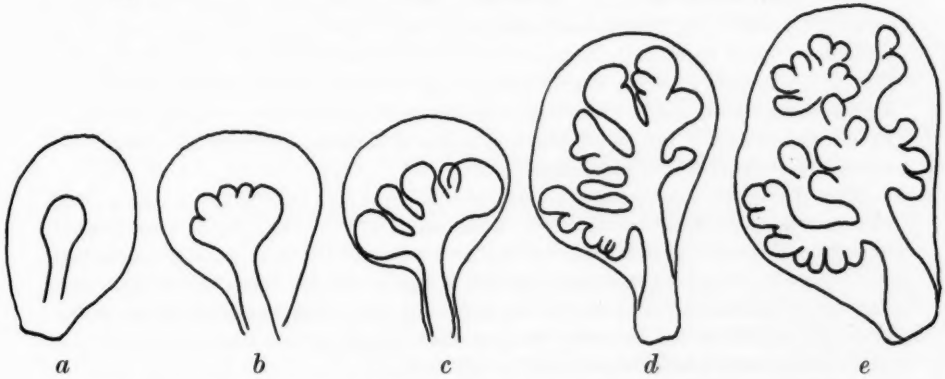
In general, the histological differentiation of the explants was very similar to that of the normal gland. The cells composing the central region of a primitive acinus were at first loosely arranged with irregular spaces between them, but as the cells multiplied they became more compact. In a few acini small cavities appeared in this compact mass similar to those which in normal development form the lumen; these were surrounded by a regular layer of epithelium.

The main duct, at first a solid cord, became hollow very soon after explantation

(Pl. 1, fig. 1). In some of the intermediate ducts a split, closed at both ends, appeared near the acini and shortly afterwards the ducts were invaded by a cavity spreading from the main duct. The lumen finally extended, but more slowly, into some of the acini, where it joined the cavity already formed in the inner cell mass. The ducts were lined, by an inner layer of columnar and an outer layer of flattened cells as in the embryo. The best developed rudiments attained a degree of differentiation about equivalent to stage 6 (p. 289) in the normal embryo, i.e. a few acini had acquired



Text-fig. 1. Camera lucida drawings of the capsule and epithelial anlage of a submandibular gland, explanted at the single bud stage and cultivated for 3 days. The rudiment underwent an average degree of development *in vitro*. *a*, when explanted, *b*, after 1 day, *c*, after 2 days, *d*, after 3 days. ×52.



Text-fig. 2. Camera lucida drawings of the capsule and epithelial anlage of a submandibular gland, explanted at the single bud stage and cultivated for 4 days. This rudiment developed exceptionally well in culture. *a*, when explanted; *b*, after 1 day; *c*, after 2 days; *d*, after 3 days; *e*, after 4 days. ×52.

cavities though most were still solid (Pl. 1, fig. 2). There was slightly more scattered cell degeneration in the acini of the explants than in those of the normal rudiment, and the ducts sometimes became abnormally dilated. This dilatation was greatest in the main duct, and in some explants the wall lost its ordinary two-layered structure and became stretched into a single layer of flattened epithelium. The lumina of the ducts often contained loose cells desquamated from the epithelium.

The sublingual gland developed in a few cultures only, as its rudiment was only occasionally included in the explants. In such cases its development was similar to,

but always less advanced than that of the submandibular. The acini were less numerous and always solid; the intermediate tracts were seldom recognizable and the main duct, which acquired a lumen, was dilated near its opening.

II. *Rudiments at the stage of early branching* (*stage* 4)

The explants were obtained from nine litters, six of which were at the 13th and three at the 14th day of gestation as calculated from the appearance of the copulation plug. Forty-six glands were cultivated, fifteen by the watch-glass method and thirty-one in hanging-drop preparations. Of these, thirty-six had four to five buds when placed in culture and the remaining ten had about 10 buds each. Three explants were rejected and so the following account is based on forty-three living cultures which were observed and drawn daily. Forty explants were fixed at intervals of 1–7 days; twenty-eight were serially sectioned and twelve mounted whole. The remaining three cultures were lost in the process of embedding.

The entire organ, including the ducts, was solid at the time of explantation. The sublingual gland, enclosed in the same mass of capsular tissue, was present as a flask-shaped structure with a single terminal bud which sometimes showed clefts indicating the beginning of branching. In nearly all the cultures the submandibular and sublingual glands were grown as a single explant. The sublingual gland developed an abnormal, polycystic structure if separated from the submandibular.

The explants of this group developed much more rapidly than those of the previous series (Pl. 1, figs. 3–5). The buds of both glands multiplied so quickly that it was soon difficult to follow them in the living cultures, but the development was rather slower than in the normal embryo. The adenomeres gave rise to well-developed lobules and intermediate tracts, most of which became hollow; the total size of the gland seemed to increase (cf. Pl. 1, figs. 4, 5), but this apparent enlargement may have been due merely to expansion and flattening.

After the fourth day the acini became so numerous and superimposed that a clear picture was no longer obtainable from whole mounts. When the glands were studied in section it was found that, as in normal development, the acini usually diminished in size as they increased in number (cf. Pl. 1, figs. 6 and 7). From this it appeared that the branching of the acini on the one hand and cellular multiplication on the other did not follow the same rhythm, but that branching took place before the size of the acinus could be reconstituted by cell division.

Histological differentiation followed a comparatively normal course. In many explants the ducts acquired lumina and cavities appeared in the acini (Pl. 2, figs. 8, 9). As in the embryo, the cavities were formed more quickly and regularly in the sublingual than in the submandibular gland. Both mitosis and scattered cell degeneration were present in the acini.

In some cultures the first signs of secretion were to be seen at about the 4th day of development. It nearly always appeared in a few scattered isolated cells bordering the central cavity of the more advanced acini. The nucleus of the secreting cells was basal and slightly compressed. The cytoplasm which had an alveolar or spongy structure, stained blue with azan or metachromatically violet with haematoxylin. These staining reactions are of no histochemical significance, however; azan produces a blue coloration not only in mucous cells, but also in the serous granules of the

submandibular acini, at least in man, rabbit, guinea-pig (Heidenhain, 1920) and mouse (Nodi, 1948; Tupa, 1926). Tupa (1926) noted a metachromatic staining with haematoxylin and a positive reaction with mucicarmine in the submandibular acini of both rat and mouse, although in these species the gland is recognized as serous (Ranvier, 1886; Löwenthal, 1894).

Unfortunately there was not enough material for special histochemical tests of the explants, but the alveolar structure of the cytoplasm and the absence of any secretory granules suggested that the secretion was mucous rather than serous. This was supported by the fact that cells of the same type were abundant in the adjacent sublingual gland of the same explants; in the normal animal the sublingual is known to produce a mucous secretion (Chievitz, 1885; Ranvier, 1886). In the submandibular gland the appearance of mucous cells seems to be a peculiarity of the gland in culture, as such elements were not seen in the normal development of the organ. As indicated above, these cells were few in number and were scattered singly in the epithelium. No sign of serous secretion was seen in the cultures.

The glands continued to develop. In the more advanced cultures the distinction between the acini of the submandibular and the sublingual glands was very clear by the 5th day *in vitro*. In the latter glands the acini had rather large cavities lined by a single layer of regular epithelium, with basal nuclei and alveolar cytoplasm which stained blue with azan (Pl. 2, fig. 10); in slides stained with haematoxylin some cells were more alveolar and distended than the rest and had a metachromatic violet coloration (Pl. 2, fig. 11). The submandibular acini had smaller, irregular cavities and, as before, the secretory cells were rather scattered.

The ducts, especially in the sublingual gland, became much dilated during the 5th and 6th days of cultivation, and many cells were detached from the walls and fell into the lumina where they degenerated. Sometimes the dilatation was extreme (Pl. 2, fig. 12); in such explants the acini were usually smaller than usual.

III. *Rudiments at an advanced stage of branching* (*stages* 5–7)

Eight cultures were made of the combined sublingual and submandibular glands from 15-day embryos which had reached stage 5 of normal development; in addition, two sublingual glands were explanted separately. The eight explants of the combined glands were fixed at intervals of 1–6 days. At this stage of development the structure of the glands is so complicated, that little could be gained by studying the living rudiments. The following observations therefore refer to sections only.

At the beginning of cultivation the acini were solid, but a cavity had already appeared in some after 2 days' cultivation. In most cultures the ducts began to dilate soon after explantation; by the end of the 5th day the distension was usually very great and the acini were pushed against the periphery of the gland. At this stage some of the submandibular acini were still solid, while others had a small or large irregular cavity. In the sublingual gland some of the acini were lined by a regular layer of alveolar cells stained blue with azan and enclosing a small round cavity. The acini of both glands contained mitoses and a few degenerate cells.

Although in most of the explants the ducts were greatly distended, there was considerable variation in the degree of dilatation in different specimens. Thus two glands cultivated for 6 days showed no distension of the ducts.

The two isolated sublingual glands were grown for 4 days and became a mass of large cavities. Both mitoses and degenerate cells were rare; very occasionally signs of secretion were observed in some of the stretched acini.

Three litters were used for cultures of 16-day glands, but there was considerable variation in the degree of development in the different litters. In one litter the submandibular glands had reached stage 6 (Pl. 2, fig. 13). Three explants were made from these embryos; one was fixed at the 3rd day and mounted whole, and the remaining two were cultivated for 5 days and sectioned. The rudiments grew profusely and formed hundreds of acini. In the two sectioned glands (Pl. 2, fig. 14) most of the acini, which were solid at the time of explantation, could be seen to have acquired cavities which were larger and more regular in the sublingual than in the submandibular. Mucous-secreting cells, of the type described above, differentiated and were particularly numerous in the sublingual acini. The ducts and a few of the acini were dilated. The lumen of both intermediate and main ducts contained a homogeneous material which may have been clotted secretion.

Seventeen cultures were made of rudiments at stage 7 (Pl. 3, fig. 15). They were derived from two litters of the same degree of development. Four of the explants contained both submandibular and sublingual glands, six the submandibular only and seven the sublingual only. All were grown in watch-glasses. Ten of the explants were fixed at intervals of 1–4 days; of these one was mounted whole and the rest serially sectioned. Some of the sections were stained with thionin or mucicarmine.

In the explants containing both glands, secretory cells developed in the acini as before and were stained metachromatically violet with thionin. In sections of a 5-day gland stained with mucicarmine (Pl. 3, fig. 17) there was a very clear difference between the sublingual, in which nearly all the cells acquired a pink colour and the submandibular, in which only a few scattered cells took the stain. On the other hand, in sections stained with azan, there were many cells with a blue coloration both in the submandibular and in the sublingual gland. The ducts remained narrow immediately behind the acini and had the usual two-layered structure, but the lumen suddenly widened into an irregular cavity lined by cylindrical epithelium. These ducts in turn communicated with still more dilated structures with a wall of flattened cells (Pl. 3, fig. 16). Dilatation was often more pronounced in the sublingual than in the submandibular gland.

The seven explants of isolated sublingual glands all became transformed into a polycystic mass in which little glandular structure was distinguishable. All the cavities were lined by a flat epithelium and there was no recognizable difference between acini and ducts. A similar extreme dilatation was also produced when small fragments of the submandibular gland were cultivated, instead of the complete organ. It therefore appeared that the enormous distension of the isolated sublingual gland was probably due to the fact that the explant was below the optimum size for normal development, and was not attributable to some peculiarity of the sublingual gland.

IV. *The development in the explants of the connective tissue capsule and stroma*

As described above, the epithelial anlage of the submandibular gland is covered by a relatively large, well-defined capsule of connective tissue which was explanted

along with the epithelial component of the organ. Its development *in vitro* was in some respects normal, but it also displayed certain special characters not seen in the embryo.

The normal development of the connective tissue in the embryonic submandibular gland has been studied in the pig by Flint (1902–3*a*, 1903) and by Moral (1913), and in the mouse by Moral (1915–16). As already stated, the sublingual gland has no separate capsule but penetrates that of the submandibular (Moral, 1915–16).

Both Flint and Moral noted that the cells of the capsule form concentric layers around but at some distance from the acini and secretory ducts; the intercellular fibres develop with the same orientation. According to Flint the concentric arrangement of the tissue is the first stage in the formation of the septa. Bertelli (1931) observed a similar concentric orientation of the mesenchyme in the development of human salivary glands.

Moral suggests that this orientation is due to an interaction between the epithelium and the connective tissue, and that the epithelium probably influences the connective tissue by both traction and pressure. Similarly, Flint regards the concentric lines of cells and fibres as representing the lines of force produced in the connective tissue syncytium by the tension of the growing epithelium. Moral considers that during the later stages of development the septa, originally formed under the stimulus of the epithelium, exert an influence on the growth of the latter.

In the cultures the phenomena recorded below were common to explants made at all phases of development, and the observations will therefore be described without particular reference to different stages. In the youngest rudiments, i.e. those explanted at the single bud stage or when only a few branches had been formed, the capsule consisted of compact cells without any orientation and no fibres were demonstrable with azan. In these cultures the histological structure which appeared during cultivation had differentiated *in vitro*, but in the more advanced explants the changes which took place in culture may have been merely a transformation of a pre-existing structure.

In all the cultures the capsule enlarged but at the same time its opacity rapidly diminished. This had the practical advantage that the epithelial branching could be very readily followed in the living explants; in fixed preparations clear images of the epithelial tissue could be obtained in whole mounts (Pl. 1, figs. 4, 5), which was not possible in similar preparations of the normal gland at the same stage of development. The sections showed that this translucence of the connective tissue in the living cultures was due to an abnormal increase in the amount of amorphous intercellular substance so that the fibroblasts were farther apart than in normal development.

The concentric orientation of the connective tissue cells round the epithelial structures, which is seen in the embryonic gland *in vivo*, was much more conspicuous in the explants (Pl. 3, fig. 19). It did not appear in all cultures but seemed to be correlated with the regularity and completeness with which the epithelium developed. Thus the best cellular orientation, in which the layers of connective tissue cells were most numerous, distinct and regular, was found in explants which had formed many acini, often grouped in lobules, and in which the ducts were not greatly dilated. In these cultures the development of the connective tissue closely resembled the normal and the cells formed concentric layers around the acini, groups of acini and the

excretory ducts; the layers were even more obvious than in the embryo, owing to the abnormal swelling of the intercellular substance mentioned above. The concentric structure sometimes faded away at a certain distance from the epithelium and the cells lost their orientation.

The rudiment of a septum was sometimes formed where two concentric systems belonging to two adjacent acini or groups of acini met and produced a thickening in the stroma (Pl. 3, figs. 19, 20). A similar thickening of the connective tissue was formed at the surface of the capsule and delimited the explant from the peripheral zone of outgrowth.

Where several concentric systems came in contact, a kind of node appeared from which the lines of cells surrounding the different acini radiated (Pl. 3, fig. 19). Sometimes the centre of this node was occupied by a cell and sometimes by a space, probably of vascular origin. This type of structure was not observed in the normal embryonic gland; it may represent, however, merely an exaggeration of a normal formation which in the embryo is obscured by the greater density of the stromal tissue.

Preparations stained with azan showed a fine network of fibrils in the stroma, and in more advanced cultures there were in addition large bundles of fibres. These observations are similar to those of Palumbi (1943).

It was evident that the cellular orientation was closely associated with the development of the epithelium which was probably the causal agent, though this cannot be established with certainty. Flint (1902–3*a*, 1903) and Moral (1913, 1915–16) suggested that in normal development the orientation of the connective tissue is due to mechanical factors such as traction and pressure. In the body the gland is closely surrounded by other tissues and organs so that the capsule of the developing rudiment is compressed externally by adjacent structures and internally by the rapidly growing epithelium. In the cultures, on the other hand, the gland could enlarge without meeting any external resistance, which possibly accounted at least in part for the abnormally large distances between the cells. In spite of the absence of any pressure from outside, however, the orientation of the connective tissue was even more conspicuous in the explants than in the normal organ. Thus any mechanical influence of the epithelium on the cells must operate at a distance through the intercellular substance. The process of orientation is therefore not a simple compression of cells against one another. The developmental relationship between the epithelial and mesenchymal constituents of the gland has been the subject of a special study which will be reported on another occasion.

At the time of explantation, even at the earliest stages of development, the capsular rudiment was richly supplied with small blood vessels (Moral, 1915–16), consisting of a single layer of endothelium. In the cultures the primitive vessels gave rise to strands of elongated cells, sometimes accompanied by bundles of fibres stained blue with azan; they usually ran between two acini. Sometimes they were hollow and the lumen contained blood cells (Pl. 3, fig. 21) which had survived from the time when the culture was made; the formation of new blood cells was not observed *in vitro*. In explants in which the epithelium had developed very readily, the major ducts were accompanied by two vessels, one on either side; the largest ducts were associated with two vessels on either side exactly as in the normal gland. The vessels

were farther from the ducts than in the normal rudiments, owing to the swelling of the intercellular substance in the explants.

Another type of structure, possibly of vascular or lymphatic origin, also appeared in the explants during the first few days of cultivation. This was a system of thin-walled lacunae (Pl. 3, fig. 22) which resembled those spaces which in normal development coalesce to form lymphatic vessels (Pensa, 1933). Whether these lacunae were identical with the rudiments of blood or lymph vessels is not known.

V. *The behaviour of the ganglion cells in the explants*

In nearly all the cultures strands of ganglion cells extended along the main ducts. They corresponded to the ganglion cells described by Moral (1913, 1915–16) in the submandibular gland of the embryonic pig and mouse and which, according to this author, are derived from the *ganglion sublinguale* belonging to the territory of the lingual nerve.

These cells were present in all cultures in which a sufficiently long section of the main duct had been preserved, regardless of the age of the culture or the stage of development at which the gland had been explanted. No mitosis and very little degeneration was seen among the ganglion cells.

The cells of the capsular tissue showed no orientation around them. Similarly, Moral (1915–16) found no orientation around the ganglion cells in normal development. This is probably due to the absence of growth in the nerve cells.

DISCUSSION

In general, the development in culture of the submandibular and sublingual glands is very similar to that in the normal embryo. Thus the epithelial buds of the explants subdivide by successive dichotomies, and differentiate into acini and ducts, the ducts acquire cavities which in the more advanced explants penetrate the acini and finally secretion begins.

There are, however, certain differences between development *in vitro* and *in vivo*. When the buds are explanted at an early stage (stages 2 and 3) they develop much more slowly than *in vivo* so that in 4–5 days only a few dozen buds at most are formed in culture, whereas a similar gland left in the embryo for the same time produces thousands of acini (cf. Pl. 1, fig. 1; Pl. 3, fig. 18). The histological development is correspondingly retarded *in vitro*. Thus at the 17th day of gestation the formation of a cavity in the acini is well advanced though not yet complete, while in a 13-day explant cultivated *in vitro* for 4 days all the acini are still solid.

The slowness of development made it possible to follow in detail the formation of the acini in the living rudiment. These observations provide direct evidence in support of the adenomeron theory, which Heidenhain (1921) based on results obtained from the examination mainly of adult glands and of one embryonic stage. According to this theory the morphological unit of a gland is its terminal bud which by repeated dichotomous division forms the glandular tree. As described above, this process, which Heidenhain deduced from fixed material, can be watched from hour to hour in the living rudiment *in vitro*.

Glands explanted at a slightly older stage (about 24 hr. later) when four to ten acini have already been formed branch almost, though not quite, as rapidly in culture

as in the embryo, and in 2–4 days produce a mass of acini which in histological structure differ little from those of the normal gland growing *in vivo* for the same length of time. The main difference between the normal and the cultivated glands lies in the distribution of the acini (cf. Pl. 1, fig. 7; Pl. 3, fig. 18); in the explants the acini were separated by wider areas of connective tissue and tended to be grouped near the periphery, whereas in the normal rudiment the lobules are evenly scattered throughout the section. Mucous secretion appears in the sublingual gland at about the same time *in vitro* and *in vivo*, and a group of secreting acini in an explant is histologically indistinguishable from a group in a normal gland of about the same age or slightly younger. The chief abnormality which appears during cultivation in glands explanted at this stage is the dilatation of the duct.

Glands explanted at a stage when several dozen acini are present, also grow and differentiate at an almost normal rate, but dilatation of the ducts is more frequent and pronounced. Sometimes only the main duct is distended, but in other cultures the intermediate ducts and even the acini also become swollen.

In all the explants degenerate cells showing pycnosis and nuclear fragmentation are present. They occur in the acini but are extremely rare in the excretory tract. Their distribution appears to be quite independent of local environmental conditions in the explant, and they are scattered among the healthy cells in both the peripheral and central acini. Similar degenerative cells are seen in the normal gland but are few in number; they are much more numerous in the explants. It seems probable therefore that this type of cell degeneration in the culture represents an exaggeration of the normal phenomenon of morphogenic degeneration (Glücksmann, 1930).

It might be thought that the cell degeneration is correlated with the formation of the cavities in the acini, but there is no evidence of this. Degenerative cells are rarely found adjacent to the developing cavities and usually the latter form without any degenerate cells being present. On the other hand, necrotic elements may appear at the periphery of the acini where no cavity is formed. The cavities seem to develop not by the death of cells but by their moving apart.

As described above, the ducts tend to dilate during cultivation. This abnormality begins in the main duct and spreads to the intermediate tract where it may stop at some distance from the acini or may extend into them. The distension is moderate and appears rather late in cultures of the early rudiment, but begins early and progresses rapidly in explants of older glands; there is, however, great individual variation among explants of the same group. The cause of this dilatation remains obscure. It has no relation to secretion, at least of the acini, and the ducts seem to be filled with a thin fluid which leaves no trace in sections and possibly filters through the walls of the ducts. The walls of the dilated ducts are not degenerate and the cells continue to divide.

SUMMARY

1. The rudiments of the submandibular and sublingual glands of *Mus musculus* were removed at various stages from the 13th to 16th day of gestation and cultivated *in vitro*.

2. Rudiments were explanted at the stage when the submandibular gland had formed the anlage of the main duct and a single bud, while the sublingual gland was only just distinguishable (usually at the 13th day of gestation). The explants formed

a variable number of acini and intermediate ducts, but always much fewer than normal; most of the acini remained solid but a few acquired a cavity. The ducts became hollow and sometimes dilated moderately. Development was much slower than in the embryo.

3. The most normal development was obtained from rudiments in which the submandibular gland had produced 4–10 buds at the time of explantation. In such explants flourishing trees of ducts and acini were formed *in vitro* at almost the normal rate; the originally solid ducts and acini became hollow in both the submandibular and sublingual glands, and in the latter what appeared to be an active mucous secretion was seen; isolated mucous cells were also present in the submandibular acini. In some cultures the ducts became abnormally dilated.

4. Other explants were made at later stages (15th–16th day of gestation) when the glands already had many buds. They continued to branch profusely during cultivation, and many acini in the sublingual gland and scattered cells in the submandibular produced mucous secretion. The excretory ducts and sometimes the acini also became extremely dilated.

5. The sublingual and submandibular glands developed more normally when explanted together in their common capsule than when dissected apart and cultivated separately. The separated glands, especially the sublingual, became transformed into a polycystic mass by extreme dilatation of the ducts.

6. A similar dilatation took place when small fragments of the submandibular gland were explanted *in vitro*.

7. In explants in which the epithelium developed normally, the mesenchymal cells became orientated in concentric lines around the acini and around groups of acini. Where two concentric systems met, a thickening was formed which represented the rudiment of a septum. The orientation resembled that in the normal gland, but was more obvious in cultures owing to the greater quantity of the intercellular material in the latter.

8. Cavities appeared in the connective tissue of the explants, which were similar to those which in normal development unite to form blood and lymph vessels. Pre-existing blood vessels became transformed into strands of elongated cells running between the acini.

9. Ganglion cells were present in all explants which contained a sufficiently large segment of the main duct.

10. Observation of the living cultures provided direct evidence in support of Heidenhain's adenomeron theory of glandular development.

I wish to thank Dr H. B. Fell, who gave me hospitality in her laboratory, suggested the subject of the research, and assisted me with helpful suggestions and criticism. Also I thank Mr V. Norfield and Mr L. King for technical help, and Mr G. Lenney who took some of the photographs.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Section of a culture, explanted when the submandibular gland was at the stage of a single bud, and cultivated for 4 days. *a*, dilated duct of the submandibular gland associated with large solid buds of irregular shape; *b*, duct of the sublingual gland associated with a small epithelial thickening, which represents the only acinus which developed. Susa; haematoxylin, eosin. ×70.

Fig. 2. A submandibular gland explanted at the single bud stage and cultivated for 5 days. A group of solid acini is connected by hollow intermediate tracts with the greatly dilated main duct. Susa; haematoxylin, eosin. ×240.

Fig. 3. Camera lucida drawing of the normal anlagen of the submandibular gland (left) and sublingual gland (right), at the stage in which the submandibular gland has begun to branch. ×52.

Fig. 4. A submandibular gland (left) and sublingual gland (right), explanted at the stage shown in Fig. 3, and cultivated for 2 days. Whole mount. Zenker; carmalum. ×48.

Fig. 5. A submandibular gland (left) and sublingual gland (right), explanted at the stage shown in Fig. 3, and cultivated for 4 days. Whole mount. Zenker; carmalum. ×48.

Fig. 6. Section of a submandibular gland, explanted at the stage shown in Fig. 3, and cultivated for 3 days. The acini are large and solid; most of the ducts are hollow. Susa; haematoxylin, eosin. ×70.

Fig. 7. Section of a submandibular and sublingual gland, explanted at the stage shown in Fig. 3, and cultivated for 6 days. The numerous acini are small and hollow; the intermediate ducts are hollow and the main ducts are slightly dilated. Susa; haematoxylin, eosin. ×70.

Plate 2

Fig. 8. Section of a submandibular gland, explanted at the stage shown in Fig. 3, and cultivated for 5 days. Cavities are forming in the acini and the intermediate duct is already hollow. Zenker; haematoxylin, eosin. ×380.

Fig. 9. The same culture as in Fig. 8, showing acini at higher magnification. Note (*a*) the mitosis (anaphase). ×600.

Fig. 10. Glands explanted at the stage shown in Fig. 3, and cultivated for 5 days. Three acini of the sublingual gland are seen. Note the regular arrangement of the nuclei and alveolar cytoplasm. Susa; azan. ×1200.

Fig. 11. Glands explanted at the stage shown in Fig. 3, and cultivated for 5 days. Three sublingual acini are seen, two of which contain cells (*b*, *c*) with an alveolar material metachromatically stained with haematoxylin. Zenker; haematoxylin, eosin. ×1200.

Fig. 12. Section of a submandibular gland, explanted at the stage shown in Fig. 3, and cultivated for 5 days. All the ducts are greatly dilated and cells are being detached from the wall of the duct in the middle of the section. Zenker; azan. ×70.

Fig. 13. Section of the normal submandibular gland from an embryo at the 16th day of gestation (early stage). The acini are solid; a hollow intermediate duct is seen. Zenker; haematoxylin, eosin. ×70.

Fig. 14. Section of submandibular and sublingual glands explanted at the 16th day of gestation (explant from the same litter as the rudiment shown in Fig. 13), and cultivated for 5 days. Very many acini are present, most of which have a cavity. *d*, *e*, clotted mass (presumably secretion) inside some ducts. Zenker; haematoxylin, eosin. ×70.

Plate 3

Fig. 15. Section of a normal submandibular gland taken from an embryo at the 16th day of gestation (late stage). Susa; haematoxylin, eosin. ×45.

Fig. 16. Section of a submandibular and sublingual gland explanted at the 16th day of gestation (late stage, explant from the same litter as in Fig. 15), and cultivated for 5 days. Note the advanced histological development of the acini and the dilatation of the ducts. Zenker; azan. ×45.

Fig. 17. The same cultures as in Fig. 16, showing two acini, both of which contain cells (*a* and *b*) which stain with mucicarmine. Zenker; haematoxylin, mucicarmine. ×1200.

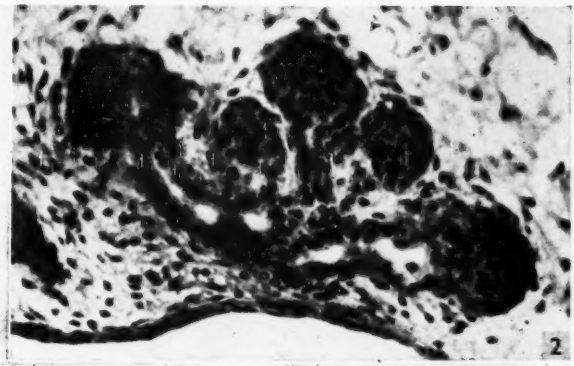
Fig. 18. Normal submandibular gland from an embryo at the 17th day of gestation. Susa; haematoxylin, eosin. ×45.

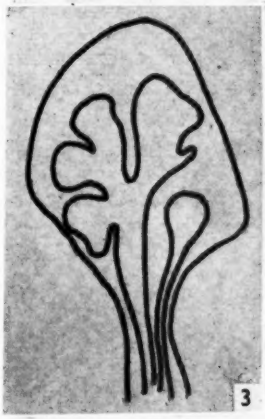
Fig. 19. Section of glands explanted at the stage shown in Fig. 3, and cultivated for 4 days. Note the concentric layers of mesodermal cells around the acini. *c*, radiating cells. *d*, formation of the septum between sublingual (right) and submandibular (left) glands. Zenker; haematoxylin, eosin. ×70.

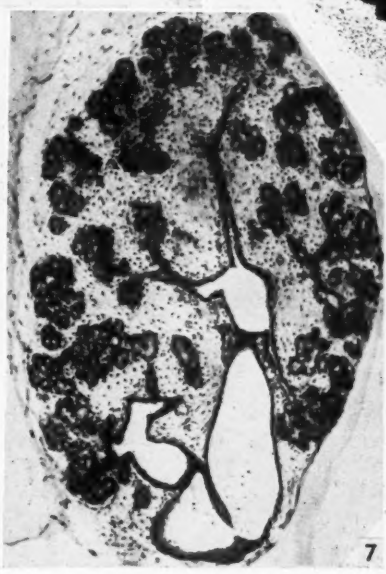
Fig. 20. Submandibular gland explanted at the single bud stage and cultivated for 4 days. Note the regular arrangement of mesodermal cells, *e*, to form the anlage of a septum. Zenker; haematoxylin, eosin. ×380.

Fig. 21. A submandibular gland explanted at the single bud stage and cultivated for 4 days. *f*, blood vessel, which continued its development *in vitro*, containing a blood cell, *g*. Zenker; haematoxylin, eosin. ×380.

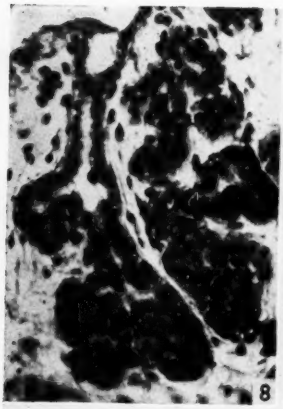
Fig. 22. Detail of a section of a submandibular gland explanted at the stage of early branching and cultivated for 6 days. Note the spongy appearance of the connective tissue. *h*, cavity with a flat cell on its wall, resembling a developing vessel. Bouin; haematoxylin, eosin. ×380.

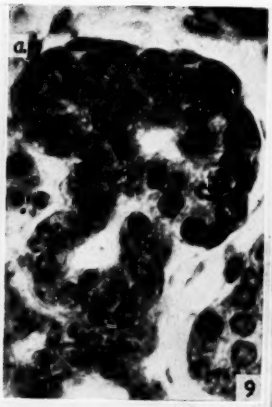


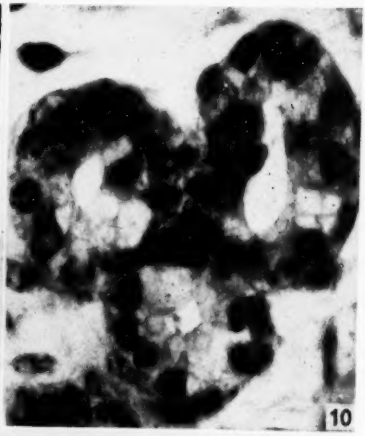


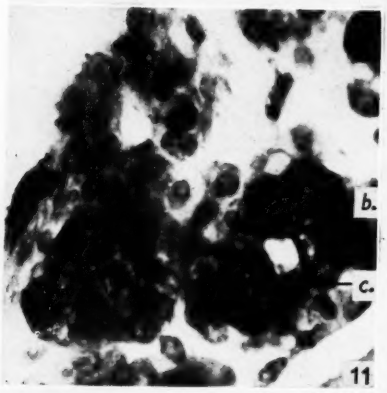


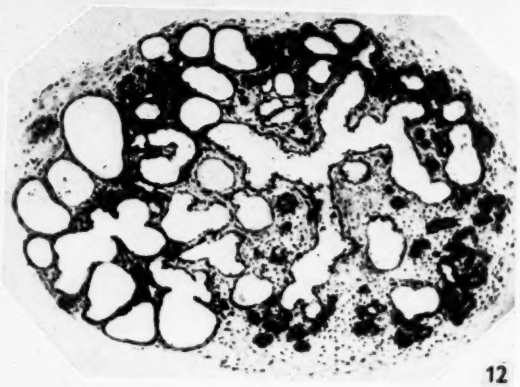
BORGHESE—SUBMANDIBULAR AND SUBLINGUAL GLANDS OF *MUS MUSCULUS*

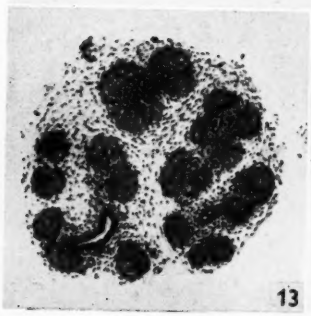


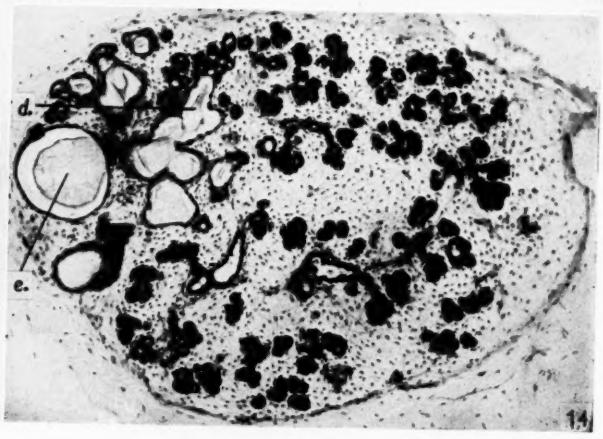




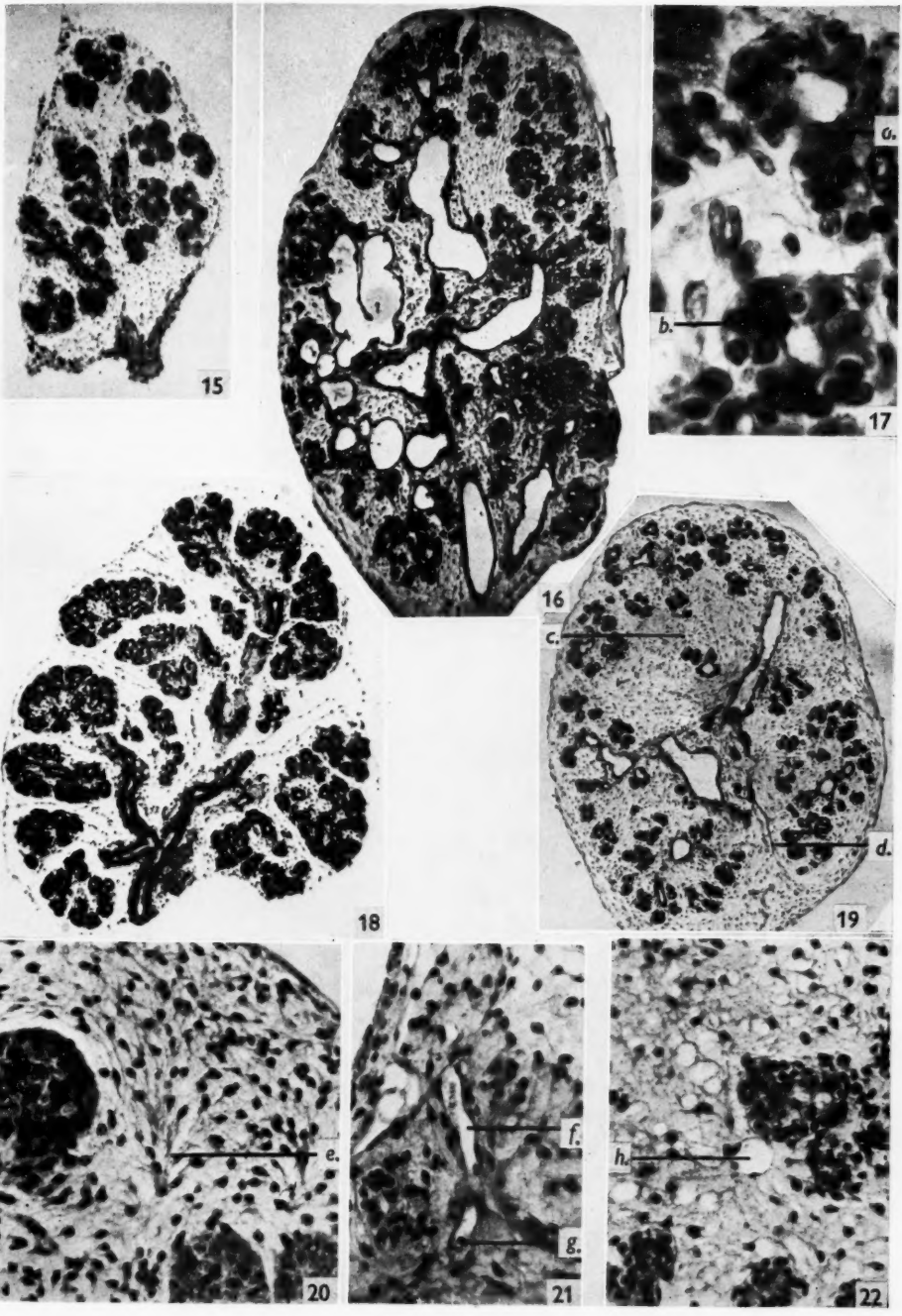








BORGHESE—SUBMANDIBULAR AND SUBLINGUAL GLANDS OF *MUS MUSCULUS*



BORGHESE—SUBMANDIBULAR AND SUBLINGUAL GLANDS OF *MUS MUSCULUS*

EXPLANTATION EXPERIMENTS ON THE INFLUENCE OF THE CONNECTIVE TISSUE CAPSULE ON THE DEVELOPMENT OF THE EPITHELIAL PART OF THE SUBMANDIBULAR GLAND OF *MUS MUSCULUS*

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INTRODUCTION

The epithelial bud, which in the mammalian embryo represents the first rudiment of the submandibular gland, is surrounded by a zone of thickened connective tissue which is quite distinct from the neighbouring tissue; this zone is termed the 'capsule', although it forms not only the definitive capsule but also the interlobular and intralobular septa and all the connective tissue around the acini and ducts.

Some hint of the existence of a connective tissue thickening around the epithelial anlage is found in certain papers dealing with the development of the gland (Chievitz, 1885; Göppert, 1906), but Kallius (1910) seems to be the first to state explicitly that the thickening is clearly delimited from the surrounding tissue.

More accurate details about the capsule and a study of its development are given in papers by Moral on the submandibular gland of the pig (1913) and of the mouse (1915–16). According to this author the connective tissue thickening in its earliest stage is 4–5 times as large as the epithelial bud, consists of round, closely packed cells and is surrounded by a region with very few cells.

Similar observations were made on human embryos by Heidenhain (1921), Fischel (1929), Löwenkron (1930), Bertelli (1931), Clara (1933–4) and Streeter (unpublished observations personally communicated by Heuser, 1949).

Moral, Fischel and Löwenkron suggest that the embryonic capsule is formed in response to a stimulus from the epithelium. According to Moral its main function is to provide a transitory filling to occupy the place destined for the glandular epithelium, which enters and invades the capsule.

The changes in the capsule during development, consisting mainly in an arrangement of the cells along concentric lines and the appearance of fibres, were studied thoroughly by Flint (1902–3*a*, 1903) and Moral. These authors suggest that there may be a reciprocal morphogenetic action of the connective tissue and epithelium; apart, however, from the merely passive function mentioned above of filling a space, the only action which is in fact defined is the orientating influence of the epithelium on the connective tissue cells and fibres, produced by pressure or some other factor acting at a distance.

In a previous research (Borghese, 1950) it was shown that the rudiment of the submandibular gland would develop almost normally when explanted *in vitro*. I therefore decided to use the tissue-culture technique to investigate experimentally the problem of the reciprocal influence of the epithelium and connective tissue on the development of the gland.

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MATERIAL AND TECHNIQUE

For the first series of experiments described below, the glands were obtained from embryos at the 13th–14th day of gestation. At this stage the epithelial constituent is flask-shaped, with a neck which will produce the main duct, and a terminal swelling from which the adenomeres are derived. The capsule is well defined, but only a very small part of it is occupied by the epithelial anlage. Earlier gland rudiments are unsatisfactory for explantation, as the connective tissue thickening is not clearly distinguishable under the dissecting binocular microscope and the epithelium does not grow well in culture.

The second series of experiments was made with glands at a later stage of development, when the epithelium had already begun to branch. Sometimes branching begins as early as the 13th day of gestation, but in other embryos it does not appear until the 14th or 15th day. Glands taken at still later stages are not suitable for such experiments because the epithelium completely occupies the capsule.

The explants were cultivated in a medium composed of equal parts of fowl plasma and extract of 11–12-day chick embryos. In the first series of experiments two cultures were grown by the watch-glass method (Fell & Robison, 1929) and two by the hanging-drop method with the tissue embedded in the medium. All the remaining explants were grown by the hanging-drop method but were placed on the surface of the clot. Each culture was observed daily and its outline drawn by means of a camera lucida.

The cultures were fixed at various intervals in Zenker's fluid or Susa. Some were stained with carmalum and mounted whole, others were serially sectioned and stained with azan or haematoxylin and eosin.

I. EXPERIMENTS ON THE UNBRANCHED RUDIMENT

(1) *The effect of removing most of the capsule on the development of the epithelium*

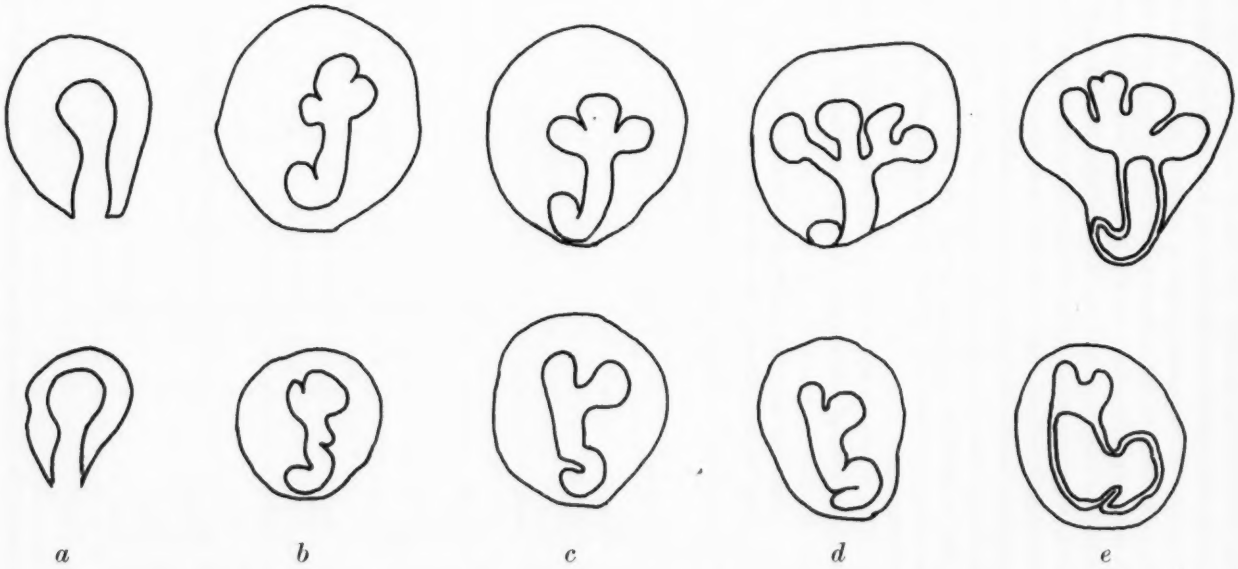
Method. Experiments were made to compare the behaviour of the epithelium in an intact gland rudiment and in one from which most of the capsule had been removed.

As stated above, in the 13–14-day gland the flask-shaped epithelial anlage extends only a short distance into the compact, oval capsule rudiment below (Pl. 1, fig. 1). Under the dissecting binocular microscope most of the capsule was removed, leaving only a thin layer of tissue around the epithelium. In the controls the capsule was left undisturbed.

The capsule was excised from twenty-five explants, but only twenty-one will be considered, because in the remaining four the controls showed early and abnormal deterioration and thus vitiated the experiment.

Results. Group 1. The cultures are divided into two groups according to the way in which the control was obtained.

Twelve cultures form the first group, in which the control consisted of the opposite gland of the same embryo grown in the same hanging-drop culture; both rudiments are at exactly the same stage in the same embryo. The material was obtained from six litters. The twelve glands and their controls were fixed after the following periods



Text-figure 1. Camera lucida drawings of the submandibular gland rudiments of the same embryo cultivated together (culture 219). The upper line of drawings shows the intact gland and the lower line the gland deprived of most of the capsule. *a*, at the time of explantation; *b*, after 1 day; *c*, after 2 days; *d*, after 3 days; *e*, after 4 days. ×52.

of cultivation: 2 days (three cultures), 3 days (two cultures), 4 days (four cultures), 5 days (two cultures), 6 days (one culture).

The behaviour of a typical pair of explants from the first group is illustrated in the text-figure. Camera lucida drawings were made at intervals of 1, 2, 3 and 4 days of two glands from the same embryo; the rudiment shown in the upper line of drawings was explanted with the capsule intact, that in the lower line was deprived of most of its capsule. The complete anlage branched regularly until by the 3rd day it had produced four buds; branching then stopped but the duct developed further and by the 4th day had acquired a medium-sized cavity. It will be seen that the duct is straight, except for a short terminal hook. The other gland, from which most of the capsule had been removed, formed only two buds which appeared later than those of the control; not only did the buds fail to divide further, but their characteristic shape had partly disappeared by the fourth day. The duct became strongly bent and by the 4th day was much dilated.

The development of the cultures is assessed according to the number of adenomeres produced at different periods of cultivation and the results are summarized in Table 1.

Table 1. *Cultures (group 1) in which the control is represented by the other gland of the same embryo*

Number of the adenomeres at different times of cultivation. Ves. = Vesicular.

			Culture period					
No.	No. of the culture		1 day	2 days	3 days	4 days	5 days	6 days
1	140	Exp.	1	1	—	—	—	—
		Control	4	5	—	—	—	—
2	143	Exp.	1	1	1	1	—	—
		Control	2	2	5	5	—	—
3	144	Exp.	1	1	Ves.	Ves.	Ves.	Ves.
		Control	1	3	3	4	8	15 (approx.)
4	145	Exp.	1	Ves.	Ves.	—	—	—
		Control	1	2	4	—	—	—
5	165	Exp.	1	2	—	—	—	—
		Control	6	6	—	—	—	—
6	167	Exp.	1	2	2	Ves.	Ves.	—
		Control	3	5	5	5	15 (approx.)	—
7	219	Exp.	2	2	2	2	—	—
		Control	3	3	4	4	—	—
8	168	Exp.	1	1	Ves.	Ves.	—	—
		Control	2	4	4	4	—	—
9	164	Exp.	1	3	3	—	—	—
		Control	2	5	5	—	—	—
10	178	Exp.	3	3	(Shapeless mass of epithelium and vesicle.)			
		Control	3	3	3	3	3	—
11	184	Exp.	2	4	—	—	—	—
		Control	3	10	—	—	—	—
12	171	Exp.	3	6	6	15–20 (approx.)	—	—
		Control	4	5	12	30–40 (approx.)	—	—

The table shows that in the first nine cultures the control glands generally produced four to five adenomeres in 2–4 days (six in one only); two, which were cultivated

longer, reached about fifteen adenomeres in 5–6 days. During the same period, five of the corresponding glands deprived of most of their capsular tissue did not ramify, three formed two adenomeres (one of these is reproduced in the text-figures) and one produced three. In all of them branching stopped early, and in several the gland subsequently lost its branches and became a small vesicle. Two cultures of this type are reproduced in Pl. 1, paired figures 2–3 and 4–5, which show the structure of the explants after 2 and 3 days' cultivation.

The remaining three cultures of this group behaved rather differently. In the 10th culture the intact control developed less than the average. In this culture both explants produced three adenomeres during the first day. After this stage the gland with the complete capsule formed only the necks of new adenomeres, and by the 4th day development stopped; the three adenomeres of the operated gland began to lose their shape on the 2nd day and deteriorated into small shapeless masses of epithelium, while the duct dilated until it was much wider than that of the control and finally formed a kind of vesicle.

In the 11th culture, the control developed abnormally well, and in 2 days formed about ten adenomeres grouped in three lobules; the operated gland, on the other hand, developed more slowly and produced only four buds.

The last pair of glands also grew to an exceptional size, probably owing to the fact that they were at a slightly more advanced stage than the others when explanted; although in each the terminal bud was still single, it showed a slight notch indicating the beginning of branching. As before, however, the control gland grew much more vigorously than its fellow from which most of the capsular tissue had been removed. In 2 days both glands formed five to six adenomeres, but by the 3rd day the intact gland had produced about twelve adenomeres, while the other showed no further development. The explants were then transplanted and both continued to grow; by the 4th day the control had produced several dozen adenomeres and a hollow but not dilated duct, while the operated rudiment had also produced a mass of adenomeres which, however, equalled a third to half the volume of the control, and a hugely dilated duct.

Group 2. The second group consists of cultures which served also as controls in other experiments described below (p. 310); for reasons which will appear later comparison could not be made with the opposite gland from the same embryo, and glands from litter-mates had to be used; since members of the same litter may not all be at the same stage of development, great care was taken to select as controls rudiments whose degree of differentiation corresponded exactly with that of the glands destined for operation.

The nine glands were fixed after the following periods of cultivation; 3 days (two cultures), 4 days (five cultures), 5 days (two cultures). The material was obtained from four litters.

The results are summarized in Table 2.

Two cultures of the first litter produced respectively five or six and ten adenomeres after 5 days' cultivation, whereas the control, although cultivated for 4 days only, had already produced about thirty to forty adenomeres. Two cultures of the second litter formed after 3 days two and seven buds, whereas three controls produced in the same time twelve, nine and four buds. Four experiments were made from the

Table 2. *Cultures (group 2) in which the control is represented by glands of littermates*

Number of adenomeres at different times of cultivation

Litter	No. of the culture		Culture period: 1 day	2 days	3 days	4 days	5 days
I	1. Exp.	172	3	4	4	5–6	5–6
	2. Exp.	173	3	5	5	10	10
	Control	171	4	5	10 (approx.)	30–40 (approx.)	—
II	3. Exp.	187	1	2	2	—	—
	4. Exp.	188	2	3	7	—	—
	Controls:	185	2	6	12 (approx.)	—	—
		186	1	3	9 (approx.)	—	—
		189	1	4	4	—	—
III	5. Exp.	220	1	Shapeless mass of epithelium			—
	6. Exp.	221	1	4	5	5	—
	7. Exp.	224	1	Shapeless mass of epithelium			—
	8. Exp.	225	2	2	2	2	—
	Controls:	219	3	3	4	4	—
		222	4	4	15 (approx.)	25 (approx.)	—
		223	2	5	7	—	—
		226	1	5	7	15 (approx.)	—
		227	1	4	11	More than 20	—
IV	9. Exp.	209	3	11	8	5	—

3rd litter. Of the six controls with complete capsules, five developed extremely well, forming fifteen to twenty-five adenomeres in 4 days, but one remained at the four-bud stage; on the other hand, none of the glands deprived of most of the capsule produced more than five adenomeres and two did not branch at all and from the 2nd day regressed to a shapeless epithelial mass.

In the single experiment made with the 4th litter no control is reported, because all the other glands of the same litter were slightly more advanced when explanted and were therefore not strictly comparable with the operated gland; they reached an advanced degree of development. The experimental explant is, nevertheless, worth describing because it showed a remarkable regression of development, a phenomenon never observed in the complete glands. At the beginning the gland grew very well and looked normal at the 2nd day, when it had formed eleven adenomeres; after this stage, however, it regressed and the adenomeres fused together until only five were present by the 4th day.

In both groups of cultures it was clear that when the gland was deprived of most of the capsule, the epithelium was confronted by a resistance to its normal expansion. This was shown by the pronounced bending of the epithelial cord which is destined to become the main duct (Text-fig. 1, lower line, and Pl. 1, fig. 6 which should be compared with fig. 7). This distortion was far greater than the small terminal hook-like bend which sometimes appeared in the duct rudiments of the intact controls, and affected a large proportion of the operated explants. The cord lengthened normally, but not finding the usual space in which to elongate, it was compelled to bend; this

is interesting in view of the fact that the capsular connective tissue is not removed from the neighbourhood of the duct but from the opposite side of the gland.

The greater variability in the development of the operated glands, as compared with that of the intact controls, may be attributed at least in part to the fact that the amount of connective tissue removed inevitably varied in different rudiments.

Histology. The question naturally arose as to whether the inferior development of the gland rudiments deprived of their capsules was due merely to injury of the epithelium during the operation. While a variable degree of trauma was probably produced, the preparations showed no more cell degeneration than was seen in the epithelium of the normal explants. There was merely an arrest of development, so that the epithelial part of the gland resembled that of a normal rudiment at a younger stage.

The histological structure of an average gland explanted after removal of most of the capsule was as follows. Even at the 3rd or 4th day of cultivation the adenomeres had a primitive structure, being solid, round and sometimes devoid of a neck. In some explants the adenomeres were composed of a superficial layer of deeply stained, cylindrical cells without either mitoses or degenerate cells, and an inner zone of more lightly stained, irregularly shaped cells with spaces between in which both degeneration and mitosis were seen. As stated above, cell degeneration was no greater than in the intact glands. Unless greatly dilated, the ducts were lined by two or three fairly regular layers of epithelial cells. As in the controls, near the opening of the duct the lumen contained a number of detached epithelial cells. When the duct was greatly dilated, as often happened in the operated rudiments, the cells of the lining epithelium were almost flat and in extreme cases were reduced to a single layer; the cavity contained detached cells.

As already mentioned, some of the glands from which the capsule was largely removed developed further than the average but later regressed. An example of such an explant is shown in Pl. 2, fig. 8. By the 2nd day this rudiment had formed eleven adenomeres but it soon began to regress, and when fixed on the 4th day the number of acini was reduced to four or five. In section they were seen to be solid, but they had a more advanced structure than that described above for a typical gland deprived of its capsule. The cells were very compact and there was no distinction between the peripheral and central cells; there was some degeneration but no more than in the controls. Each adenomere had a very distinct neck which was partly solid and partly invaded by the cavity extending from the main duct. The lumen of the main duct was not dilated, which was unusual, and was lined by three layers of cells which near the opening contained a large intracellular vacuole like the corresponding cells in the normal duct. The capsule was fairly thick, showed little differentiation and there was some concentric orientation of the cells around the acini.

Other rudiments developed less than the average; either the gland produced no adenomeres or lost them by regression and became a vesicle or a small, shapeless mass of epithelium. In such cases the histological structure was that of the main duct. If the explant was vesicular (Pl. 2, fig. 9) the epithelium was stretched and consisted sometimes of one layer and sometimes of several layers of flattened cells. Some cells projected into the cavity, some seemed on the point of detachment and others were already free and degenerating in the lumen. If the duct had originally been bent,

traces of the bends persisted as folds projecting inwards; cells in course of detachment were particularly numerous on the crests of these folds.

As described above, the epithelium sometimes formed not a vesicle, but an apparently solid mass (Pl. 2, fig. 10); this often happened if no branches developed at the beginning of cultivation. Sections showed traces of a cavity, however, and the epithelium resembled that of a main duct with an exceptionally thick wall. Most of the epithelial cells contained a large intracellular vacuole at the lower pole, as in the normal embryonic duct near its opening. The behaviour of the connective tissue in the glands from which most of the capsule had been removed differed in an important respect from that of the controls. In the latter, although there was some migration of cells and consequent spreading of the capsular tissue, this was not great and the capsule remained fairly compact. When the capsule was reduced to a thin layer around the epithelium, however, it rapidly spread into a flat, diffuse sheet of cells. It was noted that this spreading and thinning of the connective tissue was greatest in those explants in which the epithelium was reduced to a small shapeless mass, and least in those in which the epithelium showed some branching.

The results of the foregoing experiments suggest that when explanted *in vitro* the epithelial anlage of the gland retains its capacity for normal, though reduced, branching only when it can develop inside the complete capsule rudiment; when most of the capsular tissue is removed, the epithelium either fails to branch or branching stops early and often regresses.

It might be assumed, therefore, that the connective tissue has a kind of organizing action on the epithelium, which cannot develop normally in the absence of the capsular rudiment. The experiments described in the next section were made to find whether tissues other than the capsule could promote the normal development of the epithelial anlage, or whether the action of the capsule was specific.

(2) *The effect of adding other tissues to epithelial rudiments deprived of their capsules*

Method. Preliminary experiments were made to find a suitable tissue to place in contact with the epithelial rudiments after the capsule had been removed. The capsule from the opposite gland or a piece of periocular tissue were tried, but the connective tissue was too scanty. It rapidly spread into a thin layer and did not materially change the conditions of the experiment; the result was the same as in the control gland from which the capsule had been removed and to which no tissue had been added.

Fragments of limb cartilage with its surrounding tissue were then added to the rudiments. In this way a fairly solid lump of tissue was obtained which did not expand into a thin sheet. The results with this material were much more satisfactory.

It seemed interesting to study the effect on the rudiments deprived of their capsules, of adding material other than connective tissue. Experiments were therefore made in which pieces of spinal cord were added to the glands; at the stage at which it was used, the cord contains only a negligible amount of connective tissue.

In these experiments three types of explants were needed, namely (1) epithelial rudiments deprived of their capsules and placed in contact with other tissue; (2) control rudiments deprived of their capsule and not placed in contact with other

tissue; and (3) a second set of controls in which the capsular rudiment was left intact. Since each embryo has only two mandibular glands, one of the two controls had to be taken from another foetus. To minimize the effects of casual variability among embryos of the same age, individuals were selected in which the gland was at exactly the same stage of development, namely flask-shaped with a distinct neck and without the notch which indicates the beginning of branching. For each observation, both the glands of the one embryo were cultivated in the same hanging-drop culture, one being deprived of its capsule rudiment and placed in contact with other tissue, and the other being grown entire. Both the glands of the second embryo were deprived of their capsules; then other tissue was added to the one rudiment, while the other was left denuded. The third embryo of the litter was treated as the first, the fourth one as the second, and so on.

Thus the control was represented alternately by a gland with an entire capsule and one with the capsule reduced to a minimum; in each observation the two pairs were comparable, being grown in as nearly identical conditions as possible. If there were enough embryos in a litter, a culture was also made in which one gland was deprived of its capsule and the other explanted entire as described in the previous section.

Of the thirteen explants studied, cartilage was added to seven (from four litters), and spinal cord to six (from two litters). Four rudiments were fixed after 3 days in culture, eight after 4 days, and one after 5 days; seven glands were mounted whole and six were serially sectioned.

Results. Table 3 shows the number of adenomeres formed in the operated rudiments to which cartilage had been added and in the corresponding controls from which the capsule had been largely removed and not replaced by other tissue (the control 224 relates to two experiments).

Table 3. *Cultures with addition of cartilage*

Number of adenomeres at different periods of cultivation.

No. of the culture		Culture period: 1 day	2 days	3 days	4 days	5 days
(*a*) Cultures in which the addition of cartilage did not produce any effect						
Exp.	186	1	1	1	—	—
Control	187	1	2	2	—	—
Exp.	188	2	2	4	—	—
Control	188	2	3	6	—	—
Exp.	224	2	2	2	3	—
Exp.	226	1	1	1	1	—
Control	224	2	2	2	3	—
(*b*) Cultures in which the addition of cartilage produced a better branching						
Exp.	221	1	6	7	15 (approx.)	—
Control	221	1	4	5	5	—
Exp.	209	6	10 (approx.)	15 (approx.)	20–25	—
Control	209	3	11	8	5	—
Exp.	174	4	5	5	15 (approx.)	20–25 (approx.)
Control	172	3	5	6	6	6

In four cultures the operated gland showed no improvement or even a retarded development, as compared with the control glands. Two rudiments developed into a

dilated duct with vacuolated cells and three or four short-necked adenomeres; the other two formed merely an epithelial cord which in one explant was solid at the end where the adenomeres should have developed and hollow at the opposite end which corresponded with the terminal part of the main duct.

In the other three cultures, the presence of the cartilage completely transformed the situation. Not only did the glands branch more richly than their corresponding controls, but they branched much more profusely than any other explanted glands deprived of their capsules in the course of this investigation. After 4 days' cultivation one of these rudiments had formed about fifteen adenomeres grouped in four lobules, while the corresponding control from which the capsule had been removed but not replaced by cartilage, produced only five buds during the same period. The second gland differentiated even further than was usual for explants with intact capsules; it formed many adenomeres of a rather advanced histological development, in which there was no distinction between the peripheral and central cells; these were united with the main duct by rather long, hollow intermediate ducts. The third gland (Pl. 2, fig. 11) which was cultivated for 5 days, also developed vigorously, producing many adenomeres and well-formed, hollow intermediate ducts. The control gland (Pl. 2, fig. 12) of another embryo of the same litter also developed rather more than was usual for a rudiment deprived of most of its capsule, and formed six adenomeres and long, hollow ducts; this was probably due to imperfect elimination of the capsule, as indicated by the fact that the sublingual gland, which ordinarily was removed with the capsular tissue, was also present in this explant. Nevertheless, the control presented a remarkable contrast to the gland associated with cartilage (cf. Pl. 2, figs. 11, 12).

The development of the six glands deprived of their connective tissue to which spinal cord had been added, is summarized in Table 4, in which the controls represented by the glands deprived of most of the capsule without addition of other tissue, were grouped together according to the two litters used for this part of the research.

Table 4. *Cultures with addition of spinal cord*

Number of adenomeres at different periods of cultivation

Litter	No. of the culture		Culture period: 1 day	2 days	3 days	4 days
(*a*) Cultures in which the addition of spinal cord did not produce any effect						
I	Exp.	225	1	2	2	2
	Control	225	1	2	2	2
(*b*) Cultures in which the addition of spinal cord produced a better development						
I	Exp.	220	1	2	2	4
	Exp.	222	1	5	12 (approx.)	25 (approx.)
	Exp.	227	1	2	3	8
	Control	220	1	1	1	1
		221	1	4	5	5
		224	1	1	1	1
		225	1	2	2	2
II	Exp.	185	1	5	6–7	—
	Exp.	187	2	5	9–10	—
	Control	187	1	2	2	—

Only one gland to which spinal cord had been added showed no improvement as compared with its control. This specimen and its control each produced two acini and a hollow, rather bent duct, but a spherical, well-defined capsule formed round the gland associated with the cord, whereas the connective tissue surrounding the control spread into a thin sheet.

The remaining five explants to which spinal cord had been added were much better developed than the controls without the cord. One rudiment (no. 220) after 4 days' growth, had formed four adenomeres only, which is within the range of the controls, but the control gland from the other side of the same embryo grown in the same culture (no. 220) did not branch at all.

Another explant associated with spinal cord and also cultivated for 4 days formed at least two dozen well-developed adenomeres and showed very little degeneration; there were no intermediate ducts but a single hollow duct. Sections showed that the connective tissue cells had a certain concentric orientation around the adenomeres as in the normal organ and in the intact explants; there were also traces of developing blood vessels. This explant, and also the third one which reached the number of eight regularly shaped buds all enclosed in a comparatively thick, rounded mass of connective tissue were more developed than the four controls from embryos of the same litter.

The last two explants in contact with spinal cord, from the same litter, were grown for 3 days. One (Pl. 3, fig. 13) formed six or seven adenomeres and the other produced nine or ten. Both explants developed much better than the control from which most of the capsule had been removed (Pl. 3, fig. 14), although rather less than the control gland with intact capsule (Pl. 3, fig. 15).

The fact that some of the rudiments deprived of their capsules and to which cartilage or spinal cord had been added developed no better than their controls was probably due to technical reasons, e.g. imperfect union between the cartilage or cord and the gland.

An interesting difference in the behaviour of the connective tissue was observed between the rudiments to which cartilage or spinal cord had been added and the controls not associated with other explants. In the former the connective tissue often reconstituted almost as large a mass round the epithelium as the original capsule, and the normal concentric arrangement of the cells around the acini was restored. In the controls, on the other hand, the capsular remains spread into the usual thin, diffuse outgrowth described above.

II. EXPERIMENTS ON GLANDS SHOWING EARLY BRANCHING

(1) *The effect of removing part of the capsule on the development of the epithelium*

Method. In this series of experiments the glands already contained three to four adenomeres when first explanted and most of the capsule was already invaded by epithelium. Consequently, merely a small peripheral dome of unoccupied connective tissue could be removed without damaging the epithelium, so that there was only a small difference between the amount of connective tissue in the operated gland and in the untouched control.

Part of the capsule was removed from ten glands, and twelve controls were cultivated intact. Three of the operated glands were compared with controls from the same embryos; the other controls were from litter-mates at exactly the same stage of development.

Of the glands from which the capsules had been partially removed, one was fixed after 2 days, two after 3 days, four after 4 days, one after 5 days, one after 6 days and one after 7 days. One of the glands with intact capsules was fixed after 2 days, two after 3 days, six after 4 days, one after 5 days, one after 6 days and one after 7 days.

Results. Of the twelve glands with intact capsules, eleven developed almost as in the normal embryo; they branched profusely and formed adenomeres which increased in number and diminished in size as development advanced and underwent a corresponding histological differentiation. In one gland, without apparent cause, development stopped after some initial branching and the number of acini regressed.

Only three of the ten glands deprived of part of their capsules showed poor branching. In the remaining seven, the branching at first sight appeared equivalent to that of the intact explants. Four of the seven rudiments, however, differed in certain respects from the controls; the adenomeres seemed to be rather fewer than in the latter and they were also larger. Sections showed that the larger adenomeres were at an earlier stage of development than those of the intact glands, being for the most part still solid while a cavity had already appeared in the latter.

Thus the results obtained in these experiments were consistent with, though less striking than, those obtained in similar experiments on younger rudiments. Moreover, a new fact emerged, not seen in the other experiments, namely that histological differentiation was more advanced when the capsule was kept whole.

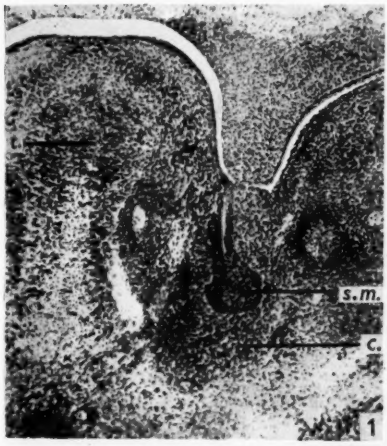
(2) *The effect of adding other tissues to glands deprived of part of their capsules*

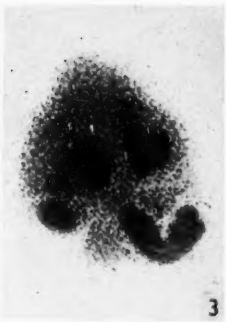
Method. Eight rudiments, from four different litters, were deprived of part of their capsules, and each was placed in contact with pieces of cartilage. Eight other rudiments, from five different litters, were cut in the same way, but placed in contact with pieces of spinal cord. Each operated gland had two controls: a gland with intact capsule, and one from which part of the capsule had been removed and not replaced by other tissue. One control was obtained from the opposite side of the same embryo from which the operated gland was taken, and the other from a litter-mate at the same stage of development.

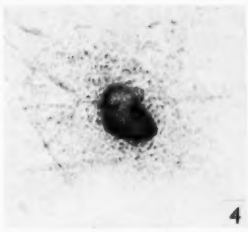
The cultures were observed and drawn daily, and fixed at intervals as follows. Those associated with cartilage: one after 3 days, five after 4 days, one after 6 days and one after 7 days; those with spinal cord: one after 2 days, six after 4 days and one after 6 days.

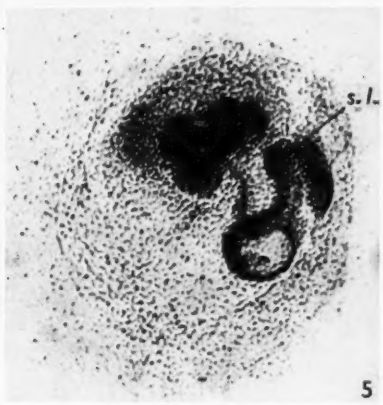
Results. There was no significant difference between the controls with intact capsules and the glands in which part of the capsules had been removed and replaced by some other tissue.

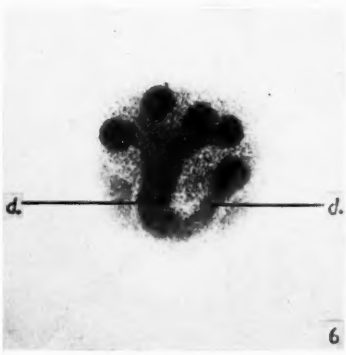
From observations on the living cultures, it appeared that six of the explants to which cartilage had been added grew and branched to about the same extent as controls from which capsular tissue had been removed and not replaced by other material; in the remaining two, it was obvious that the glands with cartilage had

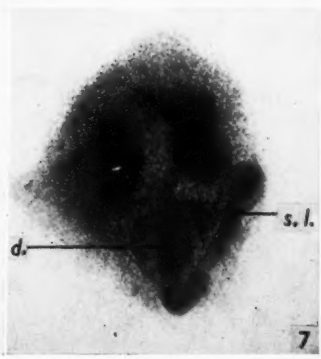




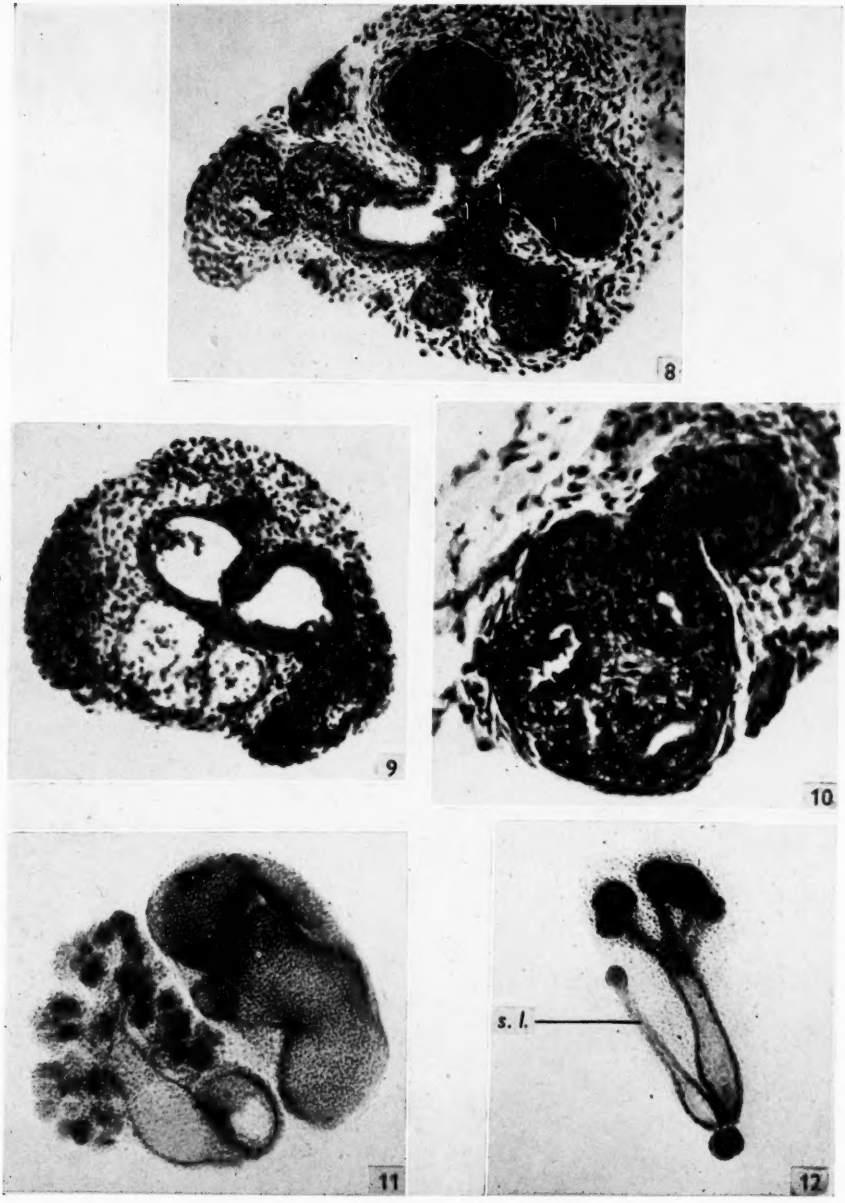




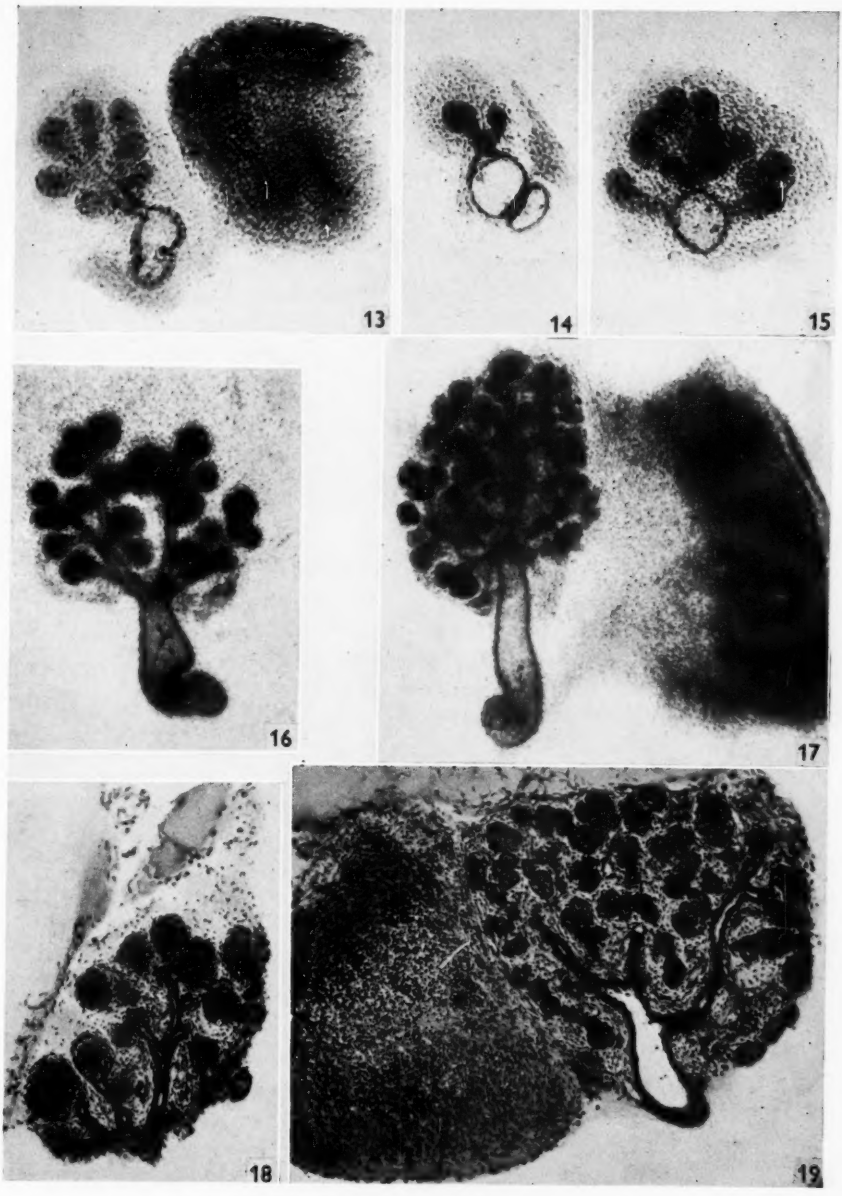




BORGHESE—EXPERIMENTS ON SUBMANDIBULAR GLAND OF *MUS MUSCULUS*



BORGHESE—EXPERIMENTS ON SUBMANDIBULAR GLAND OF *MUS MUSCULUS*



BORGHESE—EXPERIMENTS ON SUBMANDIBULAR GLAND OF *MUS MUSCULUS*

grown better than the controls without this addition, and in one of the two the adenomeres were obviously smaller than in the control. Of the explants associated with spinal cord, one grew badly and was inferior to the control without cord; three grew about as well as the controls, and in four the glands with spinal cord branched much more profusely than the controls.

In these cultures the adenomeres were so numerous and superimposed, however, that in the living glands it was impossible to count them or to see their histological structure clearly. A study of the whole mounts, and especially of the histological sections, showed that the explants deprived of part of their capsules and placed in contact with other tissues, formed more numerous and smaller acini and reached a more advanced degree of histological differentiation than the controls to which no tissue had been added (cf. Pl. 3, figs. 16–19).

These results confirmed those of the previous experiments on older rudiments, and supported the view that the presence of tissue other than the normal capsule favours epithelial differentiation. This was not shown in the experiments on the early rudiments, probably owing to the fact that the explants did not reach a sufficiently advanced stage of histological development.

DISCUSSION

We must now consider what factors are responsible for inhibiting the development of the epithelial anlage of the submandibular gland when most of the capsular rudiment is removed.

It seems unlikely that the inferior development was due solely to trauma. In the first place, as described above, there is no histological evidence of greater cellular damage in the glands deprived of their capsules than in the intact controls. In the second place, whatever damage was inflicted, it did not prevent the glands from developing almost as well as the intact rudiments when cartilage or spinal cord were added after removal of the capsular tissue.

As already stated, the degree of epithelial development attained by the glands after removal of their capsules was usually correlated with the extent to which the remaining connective tissue spread out on the medium; thus development was least when the diffuse outwandering of the capsular tissue was greatest, and most advanced when the surrounding tissue was most compact. From this it seems clear that the capsule has an important influence on the development of the epithelial anlage. In this connexion the observations of Rienhoff (1922) on the development *in vitro* of the chick metanephros are interesting. This author found that the epithelial elements only differentiated normally where the surrounding stroma remained thick and compact; when there was a diffuse outgrowth into the culture medium the rudimentary tubules failed to develop further and merely formed indifferent sheets of cells.

At first sight, the fact that, when another tissue was substituted for the missing part of the capsule, the gland developed almost as well as when the capsule was intact, suggests that the influence of the capsule is not specific. A closer examination of the results, however, shows that they do not exclude the possibility of a specific action of the capsular rudiment. As described above, when cartilage or spinal cord was added to the defective gland, the epithelium became surrounded by a much larger and more compact mass of connective tissue than in similar explants not placed in

contact with other tissue. When cartilage was added, it was possible that this capsular material might have been derived from the perichondrium, but this could not be true when pieces of spinal cord were used. At most, only a trace of connective tissue was left adherent to the cord, and it was far less in amount than that which remained attached to the glandular epithelium. It can thus be assumed that, at least when spinal cord was added to the rudiment, the new capsule originated chiefly from the remains of the original capsular rudiment.

It is also uncertain whether the added tissue promoted the growth and development of the gland by a direct action on both the capsular and epithelial constituents, or whether it had a direct action on one constituent and only a secondary effect on the other. Thus it is not known whether the regeneration of the capsular tissue was the primary effect and was responsible for the improved development of the epithelium, or whether it was the epithelium that was stimulated and by its increased growth evoked a corresponding proliferation of the connective tissue. The latter hypothesis seems improbable, but it may well be that the added tissue had a beneficial effect on both elements of the gland.

Why the addition of other tissue to the defective gland rudiment should have promoted the growth of the latter is not known. Possibly the presence of another organ provided a more normal environment for the gland. It is also possible that the increase in the total mass of the explant which resulted from the addition was beneficial. This latter view receives some support from the fact that I failed to get satisfactory development *in vitro* either from rudiments earlier than the 13th day or from small fragments of even more advanced stages (15th day and older). A certain minimum size of explant may be necessary for the normal development of the tissue.

It will be seen that the results of the experiments described in this communication are not easy to interpret, and more work is required. Although there is little doubt that, at least *in vitro*, the capsular rudiment is essential for the normal development of the epithelial anlage, there is at present no evidence as to whether the capsule has a specific organizing action, whether it merely provides the mechanical conditions necessary for the expression of developmental potencies already inherent in the epithelium or, finally, whether after removal of the capsule the epithelium fails to develop because the total mass of the explant is too small.

SUMMARY

1. Experiments were made to compare the development *in vitro* of rudiments of the submandibular gland of the mouse (*a*) when the gland was explanted intact, and (*b*) when most of the capsular rudiment was removed.

2. Intact glands explanted at the single bud stage branched in the same way as in normal development, but much more slowly, and in 3–4 days' cultivation formed four to fifteen adenomeres and a long, straight duct.

3. Similar glands from which most of the capsule had been removed developed very little; some showed no branching, others began to branch and then regressed. The duct could not extend properly and became bent and sometimes greatly dilated. The more compact the surrounding connective tissue, the better the development of the defective explants.

4. When a piece of cartilage or spinal cord was added to a rudiment deprived of

most of its capsule, the epithelium often developed almost but usually not quite as well as in the intact control, and far better than in the defective glands to which no tissue had been added.

5. In rudiments from which most of the capsular tissue had been removed, a new capsule was formed when the gland was placed in contact with spinal cord or cartilage. At least in the explants associated with spinal cord, the new capsule was formed from the remains of the old one. It is not known whether this regeneration of the capsule was the cause or the effect of the better development of the epithelium.

6. Similar experiments were made with slightly older rudiments in which branching had already begun. At this stage only a very small part of the capsule could be removed, so that the difference in anatomical development between the intact and the defective rudiments was usually slight. The addition of cartilage or spinal cord, however, improved both the anatomical development and the histological differentiation of the defective explants.

7. The significance of the results is discussed.

I wish to thank Dr H. B. Fell, who suggested the subject of this research and helped me during its progress, Dr A. Glücksmann for his advice and criticism, Mr V. C. Norfield and Mr L. J. King for their technical assistance, and Mr G. Lenney, who took most of the photographs.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Frontal section of the mouth of a mouse embryo at the 13th day of gestation. The epithelial anlage of the submandibular gland forms a single bud (*s.m.*) surrounded by a thickened connective tissue or capsule (*c.*) *t.*: tongue. Zenker; haematoxylin, eosin. ×60.

Figs. 2, 3. Submandibular gland anlagen taken from the same embryo at the stage of a single bud and cultivated together for 2 days. (cult. 165). Fig. 2: the gland from which most of the capsule was removed; it developed two adenomeres and the connective tissue is very scarce and thin. Fig. 3: the intact gland; it developed about four adenomeres and a regular capsule. Zenker; carmalum, whole mount. ×48.

Figs. 4, 5. Submandibular gland anlagen taken from the same embryo at the single bud stage and cultivated together for 3 days (cult. 145). Fig. 4: the gland deprived of most of its capsule; it became a small vesicle. Fig. 5: intact gland; it developed four adenomeres and a duct; on the right side the sublingual gland (*s.l.*) appeared and produced a bud. Zenker; carmalum, whole mount. ×48.

Figs. 6, 7. Submandibular anlagen of the same embryo, taken at the stage of a single bud and cultivated together for 2 days (cult. 184). Fig. 6: gland from which most of the capsule was removed; the duct (*d.*) is bent. Fig. 7: gland with whole capsule: the duct is straight; on the right side, the sublingual gland (*s.l.*) is seen. Susa; carmalum, whole mount. ×48.

PLATE 2

Fig. 8. Submandibular gland deprived of most of the capsule at the single bud stage and cultivated for 4 days (cult. 209). It has developed comparatively well and has formed a hollow duct and solid adenomeres. Susa; haematoxylin, eosin, section. ×120.

Fig. 9. Submandibular gland deprived of most of the capsule at the single bud stage and cultivated for 3 days (cult. 164). Note the vesicle with group of cells becoming detached from the wall and projecting into the cavity. Zenker, haematoxylin, eosin, section. ×100.

Fig. 10. Submandibular gland deprived of most of the capsule at the single bud stage and cultivated for 4 days (cult. 220). It became a very small epithelial mass, with a small cavity and intracellular vacuoles. Susa; haematoxylin, eosin, section. ×240.

Figs. 11, 12. Gland rudiments from different embryos of the same litter both deprived of most of the capsule and cultivated for 5 days. Fig. 11: associated with cartilage (cult. 174). Fig. 12: without any addition (cult. 172). Although the development of the second explant is fairly good and a sublingual gland also (*s.l.*) has formed, the development of the first is more advanced. Susa; carmalum; whole mount. ×48.

PLATE 3

Figs. 13–15. Two gland rudiments from one embryo (Figs. 13, 15) and one from a second embryo of the same litter (Fig. 14) cultivated for 3 days. Fig. 13: gland deprived of most of its capsule and grown in association with a piece of spinal cord (cult. 185); it developed much better than the gland shown in Fig. 14 (cult. 187) deprived of most of its capsule and without any addition, which gave only two small adenomeres and a dilated duct. The intact gland of Fig. 15 developed similarly to the gland with spinal cord (the duct is missing here because it was accidentally lost during explantation). Susa; carmalum, whole mount. ×48.

Figs. 16, 17. Submandibular gland rudiments taken from the same embryo at the stage of four adenomeres, both deprived of part of the capsule and cultivated together for four days (cult. 216). Fig. 16: without any addition. Fig. 17: with a piece of spinal cord. Fewer and bigger adenomeres have been formed in the first gland, more and smaller adenomeres in the second. Susa; carmalum, whole mount. ×48.

Figs. 18, 19. Two submandibular gland rudiments taken from the same embryo at the stage of four adenomeres and cultivated together for 4 days, both deprived of part of the capsule. Fig. 18: without addition. Fig. 19: with a piece of spinal cord. Fewer and larger adenomeres have developed in the first gland and the differentiation of the duct is less advanced; more and smaller adenomeres and a more advanced development of the ducts are seen in the second gland. Susa; haematoxylin, eosin (Fig. 18); azan (Fig. 19); section. ×70.

THE FATE OF THE NERVES OF THE DECIDUOUS TEETH

By A. MOHIUDDIN
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The nerve supply of the teeth has been extensively investigated. An excellent summary of existing knowledge has been given by Orban (1944), and Bernick (1948) has reviewed much of the literature, but no systematic account of the pattern of innervation of deciduous teeth has been found although there is no reason to suppose that it differs from that of the permanent teeth.

Regarding the fate of the nerves of the deciduous teeth no record has been found except that by Bradlaw (1936). This author came to the conclusion that nerves of the pulp degenerate in some cases but not in others when such teeth are being shed. He does not indicate whether the degeneration, when it occurs, precedes or follows the disorganization of the normal structure of the pulp. This is a matter of some importance. If degeneration of nerves follows disorganization of the pulp, it could well be merely a consequence of the local changes. If, on the other hand, nerve degeneration precedes the disorganization of the pulp, it is necessary to postulate more remote factors, possibly similar to those which control the development of the dentition as a whole. In view of Bradlaw's inconclusive results it is obviously desirable to reinvestigate the problem, and to trace the extent to which degeneration in nerves of the pulp spreads in a central direction, a question which Bradlaw did not consider at all.

MATERIAL AND METHODS

In five adolescent cats some of the deciduous incisor and canine teeth were found to be loose. The jaws of these animals were radiographed to confirm that these were deciduous teeth in process of being shed and also to ascertain the state of root resorption. The jaws were fixed in 10 % formol-saline and decalcified in 15 % formic acid. Blocks containing incisors and canines were cut from both jaws of each animal. Four lower blocks were treated by the Bielschowsky silver method and sectioned in paraffin at 10 μ. One lower block and two upper blocks were similarly sectioned, and several sections from each were stained by Romanes' (1946) silver method. For comparison, one lower block from a fully grown cat and one half of the entire mandible of an 8-week-old kitten were treated by the Bielschowsky method and sectioned in paraffin, the kitten's mandible being mounted serially.

The inferior dental and the maxillary nerves of the adolescent cats were also fixed in formol saline. Several transverse sections (7 μ thick) from each inferior dental nerve were stained by Romanes' method to show the axons. For comparison, similar preparations from other animals possessing sound sets of teeth only (either deciduous or permanent) were available. The maxillary nerves were stained by the Swank and Davenport modification of the Marchi method and sectioned lengthwise

* This work formed part of a Ph.D. Thesis accepted by the University of London, 1949, and was begun in St Thomas's Hospital Medical School.

serially in paraffin at 15 μ. A similar nerve from a fully grown animal and a piece of its spinal cord were used as controls.

OBSERVATIONS

The nerve fibres in the pulp of sound teeth, whether deciduous or permanent, showed a similar pattern after staining by both silver methods. Pl. 1, fig. 1, shows the arborization under the crown of a deciduous tooth, and Pl. 1, fig. 2, is taken from a similar region of a permanent tooth. For the most part the axons were of even diameter, but at high magnifications some of the finest were beaded. The nuclei of the neurilemma were either not seen at all or appeared as small fusiform vesicles. They resembled the vesicles, later to be described, in degenerating axons, but could always be distinguished because of their arrangement at regular intervals along the length of the axons, and also their much smaller size. The ultimate termination of most of the fibres appeared to be around the bases of the odontoblasts. The number of fibres which passed into the predentin was small and no fibres were seen in the dentinal tubules. In spite of the close proximity of blood vessels and nerves, no fibres could be seen terminating in the walls of the vessels. Far more nerve fibres appeared to end in the crown than in the root.

In all adolescent cats those deciduous teeth which were in the process of being shed, showed well-marked changes in the appearance of the nerve fibres within the pulp. Most axons showed an uneven calibre, although this, by itself, was not considered sufficient evidence of degeneration; it was, however, usually accompanied by other structural changes of a more definitely degenerative character. These often took the form of numerous large varicosities or vesicles in the axons and this was accepted as a definite sign of degeneration. Pl. 1, fig. 3, shows such swellings, and it is possible that the vesicle at one end of the fibre showing degenerative changes has been formed by the vacuolation within a varicosity. Indeed, the large varicosity next to the definite vesicle appears to be in the process of being converted into a vesicle. Some vesicles were disproportionately large in relation to the size of the axon bearing them, and some had a fenestrated appearance (Pl. 1, fig. 4). Segments of some of the axons had serrated edges which gave them a comb-like appearance (Pl. 1, fig. 5). In other cases the axons were fragmented either into small segments or into rows of granules (Pl. 1, fig. 6). Similar, though less marked, changes were found among the nerve fibres of the periodontal membrane.

The nature of the material does not permit classification of the various degenerative changes into well-defined stages based on the chronological sequence in which they occur. On the whole, it appears that the earliest and most persistent change is the uneven increase in the thickness of the axons. This is followed by the formation of varicosities, which are later converted into vesicles. The vesicles probably burst, leaving behind a serrated axon. Finally fragmentation into granules occurs and appears to be followed by dissolution.

The degenerative changes described above were not developed to the same degree in all the fibres in a tooth which was being shed, or even in all the fibres in a single bundle within the pulp. Some showed advanced changes, while other adjacent fibres showed none at all. For example, in Pl. 1, fig. 3, there are two extremely fine fibres besides the relatively thicker one which shows swellings, and similarly, small

lengths of some rather fine fibres can also be seen in Pl. 1, fig. 5. These fine fibres do not show any changes. On the whole, degenerative changes were demonstrable only in such axons as were about 1 μ or more in diameter. The finest axons disappeared in the later stages of root resorption without preceding changes in structure.

Some of the degenerative changes, such as the formation of varicosities, vacuolation and fragmentation, were clearly seen before the onset of root resorption, although, judging from the state of development of the adjacent permanent teeth this seemed to be imminent. In still other specimens, degenerative changes were seen in nerve fibres lying in apparently undisturbed pulp tissue, although the dentin of the root was being resorbed, and no teeth showing root resorption were free from degenerating nerve fibres. As other sound teeth in the same sections or blocks showed only normal nerve fibres, it can safely be assumed that artefacts of fixation were not responsible for the different appearance of the fibres in the teeth that were being shed. With advancing root resorption, the number of nerve fibres seen in the pulp diminished steadily, so much so that in the later stages, only occasional fragments of axons of the finest calibre could be seen, and the normal arborization of fibres had completely disappeared. The crown of the tooth then seemed to rest on granulation tissue, in which there were hardly any nerve fibres.

The inferior dental nerve trunks of the adolescent cats did not show degeneration in any axons in spite of the presence of such changes in the fibres in the teeth. There were no empty sheaths of Schwann or other signs of the disintegration of axons. Apart from the expected small size of fibres in the young cats, the appearance of the inferior dental nerve was very similar in all the animals examined. The maxillary nerves of the adolescent cats did not show any Marchi reaction in the myeline sheaths, so that no evidence was found for the spread of degeneration into the nerve trunks proximal to the jaw, either in the axon or its sheath.

DISCUSSION AND CONCLUSIONS

This investigation has shown that the pattern of innervation of sound deciduous teeth is very similar to that of permanent teeth, and that as the teeth are shed, nerve fibres more than 1 μ in thickness show degenerative changes. Fibres less than 1 μ in thickness do not show corresponding changes, but merely disappear from the pulp during the later stages of root resorption. The degeneration does not spread to the stem fibres in dental nerve trunks or the large bundles of fibres into which they divide within the jaws.

It was pointed out in the review of the literature that Bradlaw's (1936) conclusions on the fate of the nerve fibres in the deciduous teeth are equivocal. It seems that Bradlaw has attached undue importance to some apparently normal nerve filaments he found in the roots of teeth showing advanced resorption. It is well known that even when a nerve is divided surgically, there are considerable differences in the rates at which different fibres degenerate (Cajal, 1928). It is possible that the apparently normal fibres in Bradlaw's material were really the refractory elements described by Cajal, and of course, the causes of degeneration in the fibres of deciduous teeth may not affect all the fibres simultaneously as in surgical division.

Published descriptions of axonal changes in fine fibres, whether myelinated or unmyelinated, were very unsatisfactory until fairly recent times. For example,

Cajal (1928) has stated that although complex changes occur near the lesion in divided unmyelinated fibres, further away in the peripheral stump only varicosities are seen before the fibres disappear. In recent years more detailed descriptions have been published, e.g. by Speidel (1936) and Weddell & Glees (1941). These workers have shown that vesiculation accompanies the degeneration of quite fine fibres. But for the purposes of the present investigation, the observations of King, Lewinsky & Stewart (1938) on the degenerative changes in the nerve fibres of the teeth, due to nutritional deficiency, have the greatest significance. These authors describe thickening, vesiculation and serration of axons in the teeth of rats deprived of vitamin A and carotene, similar to those described in this paper during the course of the normal shedding of the deciduous teeth in cats. In addition, there was unmistakable fragmentation, similar to that observed by Bradlaw (1939) in traumatic degeneration of nerves of the pulp following fracture of the mandible.

It has already been shown that definite degenerative changes appear in some of the nerve fibres before there is any evidence of root resorption. While this investigation has thrown no light on the cause of this degeneration, it has at least established that it is not secondary to the disorganization of the pulp which follows root resorption. Similar 'spontaneous' degeneration of nerve fibres has been said to occur in the nerves of the papillae of the organ of Eimer in the growing mole (Boeke, 1940). It may be noted that this early terminal and preterminal degeneration in the nerves of deciduous teeth probably accounts for the absence of pain when they are shed.

The loss of the deciduous teeth and of the nerves supplying them might be expected to lead to a loss of fibres from the dental nerve trunks and of cells from the semilunar ganglion. The latter point was not investigated, since no evidence was found for the loss of fibres in the nerve trunks. Serial sections of the jaw of an 8-week-old kitten, stained by Bielschowsky's method, gave evidence for the branching of axons near the roots of both deciduous and permanent teeth. It seems likely that both sets of teeth are supplied by branches from the same stem fibres, so that the loss of one set of teeth need affect only some of these branches, and not the stem fibre from which they spring. If this is so, the innervation of the teeth would be very similar to that described by Weddell (1941) for the pain spots in the skin.

SUMMARY

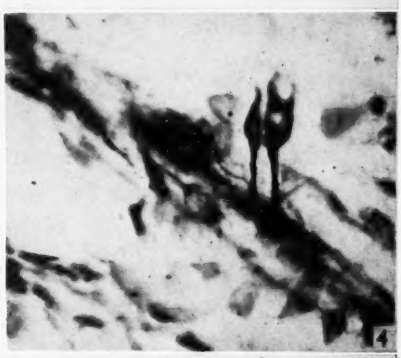
1. In the cat, nerves of deciduous teeth about to be shed, from five adolescent animals were compared with nerves of sound permanent and deciduous teeth in the same number of young and adult animals.

2. Degenerative changes could always be demonstrated in the axons in the pulp of deciduous teeth in process of shedding. These changes were confined to axons 1 μ or more in diameter; finer axons disappeared without preceding changes demonstrable by the silver impregnation methods employed.

3. The onset of degeneration in the nerves preceded evidence of root resorption.

4. Degenerative changes were found only in the axons in the pulp; they were terminal and preterminal in location and did not spread centrally to involve the axons in the dental nerve trunks.

I wish to thank Prof. F. Goldby for much advice and helpful criticism.



MOHIUDDIN—FATE OF THE NERVES OF DECIDUOUS TEETH

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EXPLANATION OF PLATE

All figures are photomicrographs of nerve fibres in the tooth pulp. Figs. 1 and 2 are taken from sound teeth and the others from teeth at various stages in the process of being shed.

Fig. 1. The normal plexus in a deciduous tooth—Bielschowsky's method, untoned. ×500.

Fig. 2. Similar normal plexus in a permanent tooth—Bielschowsky's method toned. ×500.

Fig. 3. A degenerating fibre showing four varicosities and one vesicle. Two very fine fibres appear normal. Bottom left, blood vessel. Romanes' method. ×800.

Fig. 4. Fragments of two degenerating fibres showing vesicles, one of which is very large. Bielschowsky's method, toned. ×800.

Fig. 5. A degenerating fibre of uneven diameter with serration of one margin. Bottom left, two rather fine and apparently normal fragments. Bielschowsky's method, toned. ×500.

Fig. 6. A bundle of degenerating fibres showing fragmentation. Top left, a blood vessel with some silver deposit. Romanes' method. ×500.

REVIEWS

Louis Jacobson. Ouvrages sur l'Organe Voméro-Nasal. With preface and notes by O. C. Hollnagel-Jensen and E. Andreasen. (1950. Pp. xxix + 175. 30 Kr.) Copenhagen: E. Munksgaard.

Louis (or Ludwig) Jacobson was a remarkable man, even in an age when versatility was the rule. At one time lecturer in chemistry at the Danish Royal Academy of Surgery, and surgeon-major during the turbulent years towards the close of the Napoleonic period, he was still able to make contributions of prime importance to the science of comparative anatomy. Jacobson's name is perpetuated by its association with the vomero-nasal organ and the tympanic plexus (nerve of Jacobson), which were his most outstanding discoveries; but his contributions in other fields—for example, on the renal portal circulation in reptiles and birds—mark him as a naturalist approaching the calibre of the great Cuvier himself. In the present volume Jacobson's researches on the vomero-nasal organ are collected together; previously unpublished descriptions and illustrations are presented, including a facsimile reproduction of the manuscript of the original communication of 1811 entitled (in translation) 'Description anatomique d'un organe de sécrétion dans le nez inconnu jusqu'ici'. Apart from its factual contents, which are of very great interest, the monograph can be read with profit as an example of careful and systematic original observation and lucid exposition. The preface and notes are clear and concise and the work is handsomely produced. It will be an important addition to the library of the comparative anatomist.

A. C. ALLISON

Man the Toolmaker. By Kenneth P. Oakley. (1949. Pp. 98, 41 illustrations. 2*s.* 6*d.* net.) London: The British Museum (Natural History).

History of the Primates (an introduction to the study of fossil man). By W. E. Le Gros Clark, F.R.S. (1949. Pp. 117, 40 illustrations. 2*s.* 6*d* net.) London: The British Museum (Natural History).

These two very attractively produced and moderately priced brochures have been published by the Trustees of the British Museum to replace the single *Guide to the Fossil Remains of Man* written by Sir A. Smith Woodward.

In *Man the Toolmaker* Dr Oakley gives a very well illustrated account of the evolutionary stages of palaeolithic culture.

The manner of making flint implements is well described and illustrated; the problem of eoliths is considered.

It surely cannot be intended that the guide should be read while visiting the Museum. It is not a catalogue, but anyone carefully reading it will be stimulated to visit the nearest flint-bearing locality, there to try his hand at producing some sort of an implement, and then to begin the fascinating pursuit of hunting for scrapers, arrow-heads and axes.

Though the guide contains a useful glossary there are still many terms in the text left unexplained which may be a source of difficulty to the layman. But this is a problem inherent in a short guide of this kind.

Prof. Le Gros Clark has produced a most useful précis of our present knowledge with regard to the history of the primates, and this short guide might well serve as an introduction for students taking a course in physical anthropology.

Perhaps because I am more familiar with the ground, I have the impression that this guide will be the more easily understood by those for whom it is intended. It is well illustrated with photographs and line drawings and deals with both living and fossil types of the lower primates, among which the tree-shrews are included; with the Australopithecinae; with the various forms of *Pithecanthropus* from Java and elsewhere; with the many varieties of Pleistocene man, and finally with the *Homo sapiens* in late Palaeolithic times. The book as a whole is a very clear survey of 'The History of the Primates'.

Each book carries a short bibliography.

C. M. WEST

BOOKS RECEIVED

Comparative Anatomy. By LEVERETT A. ADAMS and SAMUEL EDDY. (Pp. 520, 364 figs., demy 8vo. 30*s.* net.) London: Chapman and Hall.

Anatomy and Ballet. (A Handbook for Teachers of Ballet.) By CELIA SPARGER. (Pp. 77, 37 X-ray and other photographs, 42 drawings. 12*s.* 6*d.* net.) London: A. and C. Black Ltd.

Practical Anatomy. By W. E. LE GROS CLARK. 2nd ed. (Pp. xvi+493, 268 figs. 8vo. 30*s.* net.) London: Edward Arnold and Co.

Gray's Anatomy. Edited by T. B. JOHNSTON, C.B.E., M.D. and J. WHILLIS, M.D., M.S., 30th ed. (Pp. 1533, 1285 figs., med. 8vo, 84*s.* net.) London: Longmans, Green and Co. Ltd.

A Short Manual of Regional Anatomy. By J. A. KEEN, M.B., F.R.C.S., 2nd ed. (Pp. 164, 75 figs., demy 8vo. 10*s.* 6*d.* net.) London: Longmans, Green and Co. Ltd.

Accessory Bones of the Human Foot. (A Radiological, Histo-embryological, Comparative-anatomical, and Genetic Study.) By DYRE TROLLE. (1948. Pp. 272, 126 figs., med. 8vo, paper. 22 kr.) Copenhagen: Einar Munksgaard.

Buchannan's Manual of Anatomy. Edited by F. WOOD JONES, D.Sc., F.R.S., F.R.C.S. 8th ed. (Pp. 1616, 847 figs., demy 8vo. 45*s.* net.) London: Baillière, Tindall and Cox.

OBITUARY NOTE

We have heard with very great regret of the death, on 22 April, of Professor A. B. Appleton, sometime President of the Anatomical Society of Great Britain and Ireland. A notice *In Memoriam* will appear in our next issue.